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REMARKS

Receipt of the Office Action, mailed March 24, 2004, is acknowledged. Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and the following remarks. This amendment adds and changes claims in this application. A detailed listing is presented, with an appropriate defined status identifier, of all claims that are or were in the application, irrespective of whether the claims remain under examination.

Claims 7-12, 14-15, and 17-20 are currently amended. With respect to these revisions, applicants note that they have amended claim 8 from "A method of treatment according to claim 7" to "The method according to claim 7," in order to bring the claims into proper form. In addition, applicants have removed the limitation that the histidine ammonia lyase is not decreased in the presence of L-histidinol or a therapeutic salt thereof to remove a potential redundancy with claim 9. As such, claim 9 has been amended to change the dependency from claim 8 to claim 7. Likewise, the dependencies of claim 10 and claim 7 have been amended reflect this change. Finally, the portions of claim 8 and claim 12 directed to fragments have been removed from these claims and placed into new dependent claims 29 and 32, respectively.

Claims 28-38 are currently being added. Support for claims 28-33 can be found in the originally filed claims and on page 13, paragraph [0048] and page 15, paragraph [0053]. Support for claims 34-38 can be found in Example 9 on page 47 and in Figures 9-11. After amending the claims as set forth above, claims 7-20 and 28-38 are now pending in this application.

Objections

I. New Matter: Figure 8 is Supported by the Specification

Applicants respectfully submit that Figure 8 does not include new matter. The figure is supported by the specification. As the Examiner acknowledges, the specification describes Figure 8 in paragraph [0034], where it states that "Figure 8 is a graph depicting the effect of pH on HAL," and paragraph [0146], where it states that the "enzyme is active over a wide range of

pH, with highest activity around pH 8.2 and high activity in physiological conditions." In addition, in paragraph 4, the specification also states that "HAL demonstrates a broad useful pH range with approximately 75% of activity being retained at pH 7.2." Further, the graph in Figure 7 is similarly described as maintaining a "significant level of activity at physiological temperature conditions." See page 46, paragraph [0146].

Based on the description of Figure 8 and Figure 7, one of skill in the art would expect Figure 8 to include a graph where the enzyme activity (U/ml) is plotted against the pH. This corresponds to Figure 7, where the enzyme activity (U/ml) is plotted against the temperature. In addition, one would expect to see a relatively flat enzyme activity around pH 8.2, with the maximum being around pH 8.2 and dropping off to about 75% of the activity at pH 7.2. One of skill in the art would expect similar activity at pH 9.2. Figure 8 accurately reflects this description. Therefore, applicants submit that Figure 8 is sufficiently described in the specification and that Figure 8 does not constitute new matter.

I. New Matter: Legend of Table 1

Applicants note with appreciation that the Examiner has withdrawn his objection regarding the coordinates in Table 1 and has indicated that the table is now clear. In addition, applicants acknowledge the Examiner's objection to the addition of "on SED ID NOS: 7 or 12" to the heading of Table 1. In light of the Examiner's comments, applicants submit that the amendment, which was made to clarify the table heading, was unnecessary. Applicants, therefore, amend the specification to remove the objectionable phrase.

Rejections

I. 35 U.S.C. § 112, ¶ 2: Claim 8 is Definite

The Examiner has rejected claim 8 as indefinite due to the typographical error "and-the." Applicants have amended the claim to recite "and the" and respectfully request that this rejection be withdrawn.

II. 35 U.S.C. § 112, ¶ 1: Claims 7-20 are Supported by the Written Description

In this Office Action, the Examiner has rejected claims 7-20 for allegedly failing to comply with the written description requirement. The Examiner asserts that the specification does not teach that the claimed methods would be effective in a patient. While the Examiner acknowledges that the specification includes *in vitro* experiments employing cell cultures, the Examiner asserts that there is "no correlation between these *in vitro* methods and actual treatment of a patient."

A rigorous or an invariable exact correlation between the disclosed *in vitro* assay and an *in vivo* activity is not required. *See* MPEP § 2164.02. Instead, the question is whether a skilled artisan would accept the *in vitro* assay as reasonably correlating to an *in vivo* activity based upon the state of the prior art. *See id.* Applicants contend that the state of the art, before the earliest priority date of the application, recognized the correlation between *in vitro* plaque assays and *in vivo* anti-viral activity.

Based on the *in vitro* tests and the description in the specification, applicants submit that those skilled in the art would know that the applicants had possession of the invention recited in claims 7-11 and that the claimed methods would be effective when administered to patients. Example 11 describes the use of VERO cells to test the anti-viral activity of histidine ammonia lyase. VERO cells are derived from the kidneys of African green monkeys and are used in virus replication studies and plaque assays. *See* THE ON-LINE MEDICAL DICTIONARY (1998) (copy enclosed). In Example 11, the cells are incubated, exposed to Herpes Simplex Virus, and then incubated again. In order to release the virus from the cells, the cells are frozen and then thawed

to room temperature. The viral dilution is subsequently placed in a plaque assay. The virus can be assayed alone, and, for the purpose of comparison, the cells can be exposed to both the virus and the test compounds and assayed in the same manner.

Plaque assays are well-known in the art as assays for a virus where a dilute solution of the virus is applied to a culture dish containing a layer of host cells. After incubation, areas in which cells have been killed (or transformed) are recognized and the number of infected virus particles in the original suspension estimated. *See* THE ON-LINE MEDICAL DICTIONARY (1997) (copy enclosed). The control results can be compared with the virus plus drug assay to determine whether the drug exhibits antiviral activity. For example, in Example 11, the results of the experiments using Herpes Simplex Virus show that histidine ammonia lyase inhibits virus replication and that histidine ammonia lyase in combination with L-histidinol shows even greater inhibition of virus replication.

Those skilled in the art are very familiar with plaque assays and often employ these assays to predict whether a therapeutic compound will succeed in an actual patient. *See* Biron *et al.*, *Antimocrobial Agents and Chemotherapy* 46(8): 2365-72 (2002) (illustrating the use of plaque assays to determine the antiviral activity of the drug compound 1263W94) (copy enclosed); *see also* Lalezari *et al.*, *Antimicrobial Agents and Chemotherapy* 46(9): 2969-76 (2002) (illustrating that the same dug compound, 1263W94, is effective in humans) (copy enclosed). Therefore, applicants submit that they have sufficiently described the claimed methods through the use of accepted *in vitro* methods, such that one skilled in the relevant art would understand that they had possession of the claimed invention, *i.e.*, a method of treating a viral infection, at the time the application was filed.

Additionally, applicants submit that, based on the *in vitro* tests and the description in the specification, those skilled in the art would know that the applicants had possession of the invention recited in claim 12 and that the claimed methods would be effective when administered to patients. Example 12 describes the use of *in vitro* cancer drug screening assays to study the effect of histidine ammonia lyase on various human tumor cells lines, for example, human

ovarian cancer cell lines. In this example, the test agents are added to a culture of the human tumor cell. The end-point determination of cell growth is then determined by *in situ* fixation of the cells, followed by staining with a protein-binding dye as described in Monks *et al.*, *J. Nat'l Cancer Inst.* 83(11): 757-766 (1991) (copy enclosed). The protein-binding dye binds to the basic amino acids of cellular macromolecules and the solubilized stain is measured spectrophotometrically to determine relative cell growth or viability in treated and untreated cells. The results disclosed in Example 12 indicate that the growth of several prostate cancer cell lines and ovarian cancer cell lines were inhibited by histidine ammonia lyase.

Measurements of tumor cell growth and viability *in vitro* are well known by those skilled in the art and are often used to predict cell growth and viability of tumor cells in a patient. *See* Monks *et al.*, *J. Nat'l Cancer Inst.* 83(11): 757-766 (1991) (copy enclosed) (describing the development of the large scale anticancer drug screening model employed by the applicants), *and also* Ingber *et al.*, *Letters to Nature* 348: 555-57 (1990) (comparing the inhibition of tumor cells *in vitro* with tumor growth *in vivo*). *See also National Cancer Institute's Investigator's Handbook* (1989) (describing NCI's use of *in vitro* models to screen drug compounds) (copy enclosed). Therefore, applicants submit that they have sufficiently described the method recited in claim 12, such that one skilled in the relevant art would understand that they had possession of the claimed invention at the time the application was filed. Applicants contend that the state of the art, prior to the earliest priority date of the application, recognized the correlation between *in vitro* inhibition of tumor cell growth and *in vivo* inhibition of tumor growth.

With respect to claims 13 and 16, applicants submit that the claimed subject matter is sufficiently described by the specification and that one skilled in the art would be convinced, in light of the specification, that the applicants had possession of the claimed invention. First, applicants note that claim 13, along with claims 7 and 12 discussed above, is an original claim. There is a strong presumption that original claims are supported by the specification. *In re Wertheim*, 541 F.2d 257, 263 (CCPA 1976).

Second, with respect to claim 13, it was known, at the time the application was filed, that non-transformed cells, when incubated in a histidine-deficient medium or in a histidine analog, enter a reversible inactive state at a specific point during the cell cycle. *See* Newman *et al.*, *Anticancer Res.* 43: 4703 (1983); *see also* Warrington, *loc. cit.* 6: 451 (1986). Further, it was known that transformed cell lines have lost their ability to stop proliferation. *See* Pardee *et al.*, *Annual Rev. Biochem.* 47: 715-50 (1974). Chemotherapeutic agents interact preferentially with proliferating cells and therefore they will not interact with inactive normal cells. *See* Warrington (1986), *supra.* Finally, those skilled in the art knew that histidine ammonia lyase converts L-histidine to urocanic acid and ammonia *in vivo* thereby depleting the circulating histidine.

Accordingly, applicants explained in the specification that administering a therapeutically effective amount of a polypeptide that has histidine ammonia lyase activity to a patient along with a therapeutically effective amount of a chemotherapeutic agent or retroviral vector will selectively deplete circulating histidine in the patient causing normal cells to become inactive. *See* page 24 of the present specification, paragraph [0078]. Applicants further explain that the chemotherapeutic drugs would be less likely to react with these inactive normal cells and therefore would confer less toxicity to a patient. *See* page 24 of the present specification, paragraph [0078]. Based on this description, one skilled in the art would be convinced that the applicants had possession of the method for reducing toxicity to normal cells as claimed.

With respect to claim 16, it was well-known, at the time the application was filed, that histidine ammonia lyase converts histidine into trans-urocanic acid ("t-UA") and ammonia. Further, it was known that irradiation at approximately 310 nm causes the photoisomerization of t-UA to its cis isomer ("c-UA"). *See* Hanson *et al.*, *Proc. Nat'l Acad. Sci. USA* 95: 10576-78 (1998). Further, studies have shown that c-UA plays a role in UVB-induced immunosuppressive mediators. *See* Norval *et al.*, *Photochem. Photobiol.* 62: 209-17 (1995). Accordingly, applicants explained that administering a therapeutically effective amount of a polypeptide having histidine ammonia lyase activity generates t-UA *in vivo* and that irradiating the patient causes the photoisomerization of t-UA to c-UA, which exhibits immunosuppressive properties. *See* page 26 of the present specification, paragraph [0082]. Based on this description, one skilled in the art

would be convinced that the applicants had possession of the claimed method for delivering an immunosuppressant to a patient.

III. 35 U.S.C. § 112, ¶ 1: Claims 7-20 are Enabled by the Specification

The Examiner also rejected claims 7-20 for allegedly failing to comply with the "enablement" requirement. The Examiner does not deny that one skilled in the art would know how to make the polypeptides described in the claims; rather, the Examiner asserts that one skilled in the art would not know how to use the claimed methods to treat a patient given the *in vitro* tests.

Applicants submit that those skilled in the art would be able to use the methods of treating a patient recited in claims 7-12 based on the teachings provided in the specification without undue experimentation. As discussed above, the plaque assays described in Example 11 and the cancer drug screening assays described in Example 12 both correlate with actual treatment of a patient. Therefore, applicants have described methods for testing the efficacy of the specified compounds to treat the specified disorders. Armed with the tests and methods disclosed in the present specification, therefore, one of skill in the art would need only routine experimentation in order to employ the methods as claimed.

Turning again to claims 13 and 16, applicants submit that those skilled in the art would be able to use the methods recited in claims 13-20 based on the teachings provided in the specification without undue experimentation. Applicants note that the elements for analyzing enablement are (1) the quantity of experimentation; (2) the amount of direction and guidance presented; (3) the presence of working examples; (4) the nature of the invention; (4) state of the prior art and relative skill; (5) predictability of the art; and (6) the breadth of the claims. *Ex Parte Forman*, 230 USPQ 546 (1986). The presence of working examples is only one of the six factors that an Examiner considers when analyzing enablement. They are not necessary or dispositive. *See In re Borkowski*, 422 F.2d 904, 908 (CCPA 1970) ("a specification need not contain a working example if the invention is otherwise disclosed in such a manner that one

skilled in the art will be able to practice it without an undue amount of experimentation"). *See also* MANUAL OF PATENT EXAMINING PROCEDURE § 2164.02 (2001).

As discussed above, the underlying chemistry of the methods claimed in claims 13 and 16 is described in great detail in the specification. *See* section II, *supra*. Moreover, the claims are well-tailored to cover those embodiments that are operable based on the described chemistry. For example, in claim 16, the claim recites that the polypeptide generates t-UA *in vivo* and that the irradiating agent causes the photoisomerization of t-UA to c-UA. Polypeptides that do not generate t-UA *in vivo* and methods of irradiating that do not cause the photoisomerization of t-UA to c-UA are not within the scope of the claim. Accordingly, given the high level of skill in the art, a skilled artisan would be able to follow the steps of claim 16 by administering a therapeutically effective amount of a polypeptide having histidine ammonia lyase activity to a patient, irradiating the patient, and testing to determine whether the polypeptide generates t-UA *in vivo* and whether the irradiating agent causes the photoisomerization of t-UA to c-UA.

Since it was well-known that histidine ammonia lyase converts histidine into t-UA, one skilled in the art would expect a polypeptide having histidine ammonia lyase activity to convert histidine into t-UA. Further, since it was well known that irradiation at approximately 310 nm causes the photoisomerization of t-UA to c-UA, one skilled in the art would expect that irradiating a patient having t-UA circulating would convert the t-UA to c-UA. Furthermore, one skilled in the art would know how to test for the presence of t-UA and c-UA and how to confirm that c-UA has immunosuppressive properties as indicated by Norval *et al.*, *Photochem. Photobiol.* 62: 209-17 (1995). This testing does not impose an undue burden on one skilled in the art because it is within the skill in the art.

With respect to claim 13, the claim is narrowly tailored because it is directed to a polypeptide having histidine ammonia lyase activity that selectively depletes circulating histidine and causes growth arrest in normal cells, without affecting the growth of tumor cells. One skilled in the art could, without undue experimentation, test a given polypeptide to determine whether it depletes the circulating histidine and whether it reduces the toxicity of normal cells

from chemotherapeutic agents or retroviral vectors as claimed. This testing is within the skill in the art and is not undue.

The Examiner further asserts that the conservative substitutions in claims 8 and 12 (now claims 29 and 32) are not enabled. Specifically, the Examiner asserts that, although one of skill in the art would know how to make the variants recited in the claims, the "artisan would not have been taught how to use the variants." In support, the Examiner asserts that it is unknown what effect the conservative substitutions would have and, in fact, the substitutions could result in a loss of or change of activity.

Applicants respond that the claims as drafted require that the substituted polypeptide maintain the histidine ammonia lyase activity that it had prior to the substitution. Therefore, the Examiner's hypothetical polypeptides that have no histidine ammonia lyase activity or have an altered activity, which the Examiner asserts are not enabled, are explicitly excluded from the claims. Therefore, the effect of the conservative substitutions is known by those skilled in the art or it can be determined, without undue experimentation, by following the steps laid out in the specification.

IV. 35 U.S.C. § 102(b): Claim 7 is Novel over Roberts and Jack

The Examiner rejected claim 7 for alleged anticipation by Roberts, *et al.* or Jack, *et al.* Specifically, the Examiner asserts that both references independently disclose "administering a histidine ammonia lyase to a patient to treat carcinoma, sarcoma, and leukemia." The Examiner asserted that the claim as written was broadly drawn to a method of treatment comprising administering a histidine ammonia lyase ant that the claim is not drawn to a method for "treating a viral infection."

Without acquiescing in the rejection and without intending to abandon claimed subject matter but to expedite allowance, claim 7 has been amended for clarification. This amendment makes clear that the recited polypeptide is administered to a patient suffering from a viral infection in order to treat the viral infection. Support for this amendment can be found at

paragraph [0072]. Neither Roberts nor Jack discloses a "method for treating a viral infection" or the step of administering the recited polypeptide "to a patient suffering from the viral infection." Therefore, the references do not each teach each and every limitation of the claim and they do not anticipate claim 7. Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

V. 35 U.S.C. § 103(a): Claims 8-9 and 11 are Patentable Over Roberts in view of Brand and Rechler

The Examiner further rejected claims 8-9 and 11 as obvious over Roberts in view of Brand or Rechler. Specifically, the Examiner asserts that it would have been obvious to one of ordinary skill in the art to use the enzyme taught by Roberts in a treatment method and to purify the histidase using the methods of either Brand or Rechler.

Applicants note that claim 7 has been amended to recite a "method for treating a viral infection" and, therefore, the methods recited in claims 8-9 and 11, which depend on claim 7, are not directed to *any* treatment method. Instead, they are specifically directed to methods for treating viral infections. Roberts does not disclose a "method for treating a viral infection" or the step of administering the recited polypeptide "to a patient suffering from the viral infection." Brand and Rechler do not teach or suggest these elements and therefore they do not, in combination with Roberts, render the claimed invention obvious.

Applicants note with appreciation that claims 10 and 12-20 are free of the prior art. Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check

being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 23 July 2004

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vero cells

A cell line derived from the kidney of the african green (vervet) monkey, cercopithecus aethiops; used primarily in virus replication studies and plaque assays.

(12 Dec 1998)

Previous: vernine, vernix, vernix caseosa, vernonin, Verocay bodies, Verocay, Jose
Next: Veronal, veronica, verriulate, verruca, verruca acuminata

plaque assay

1. <investigation> Assay for virus in which a dilute solution of the virus is applied to a culture dish containing a layer of the host cells, convective spread is prevented by making the medium very viscous. After incubation the plaques, areas in which cells have been killed (or transformed), can be recognised and the number of infective virus particles in the original suspension estimated.

2. Assay for cells producing antibody against erythrocytes or against antigen that has been bound to the erythrocytes. The cell is surrounded by a clear plaque of haemolysis. Basic principle behind the assay is the same as for the virus plaque assay.

(18 Nov 1997)

Previous: planum transpyloricum, planuria, Pla protease, plaque, plaque and tartar cause
Next: plaque-forming unit, Plaque Index, plash, plashing, plashoot, -plasia

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Potent and Selective Inhibition of Human Cytomegalovirus Replication by 1263W94, a Benzimidazole L-Riboside with a Unique Mode of Action†

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Benzimidazole nucleosides have been shown to be potent inhibitors of human cytomegalovirus (HCMV) replication in vitro. As part of the exploration of structure-activity relationships within this series, we synthesized the 2-isopropylamino derivative (3322W93) of 1H-β-D-ribofuranoside-2-bromo-5,6-dichlorobenzimidazole (BDCRB) and the biologically unnatural L-sugars corresponding to both compounds. One of the L derivatives, 1H-β-L-ribofuranoside-2-isopropylamino-5,6-dichlorobenzimidazole (1263W94), showed significant antiviral potency in vitro against both laboratory HCMV strains and clinical HCMV isolates, including those resistant to ganciclovir (GCV), foscarnet, and BDCRB. 1263W94 inhibited viral replication in a dose-dependent manner, with a mean 50% inhibitory concentration (IC_{50}) of 0.12 ± 0.01 μM compared to a mean IC_{50} for GCV of 0.53 ± 0.04 μM, as measured by a multicycle DNA hybridization assay. In a single replication cycle, 1263W94 treatment reduced viral DNA synthesis, as well as overall virus yield. HCMV mutants resistant to 1263W94 were isolated, establishing that the target of 1263W94 was a viral gene product. The resistance mutation was mapped to the UL97 open reading frame. The pUL97 protein kinase was strongly inhibited by 1263W94, with 50% inhibition occurring at 3 nM. Although HCMV DNA synthesis was inhibited by 1263W94, the inhibition was not mediated by the inhibition of viral DNA polymerase. The parent benzimidazole D-riboside BDCRB inhibits viral DNA maturation and processing, whereas 1263W94 does not. The mechanism of the antiviral effect of L-riboside 1263W94 is thus distinct from those of GCV and of BDCRB. In summary, 1263W94 inhibits viral replication by a novel mechanism that is not yet completely understood.

Human cytomegalovirus (HCMV) is a herpesvirus that causes a benign infection in an estimated 40 to 100% of populations in the United States (reviewed by Sia and Patel [23]). In most cases, HCMV infection is not associated with disease; however, in patients with an immature or compromised immune system, HCMV infection can be a serious or even life-threatening disease. Four drugs—ganciclovir (GCV), its prodrug valganciclovir, cidofovir, and foscarnet—are currently used for the treatment of systemic HCMV infection; however, there are a number of disadvantages associated with each of these therapies. Cidofovir and foscarnet are available only as intravenous formulations, whereas GCV is given intravenously for initial treatment of systemic disease. With all anti-HCMV drugs currently available, there can be serious side effects associated with prolonged treatment. In addition, the drugs have similar mechanisms of action, all ultimately targeting the HCMV polymerase; therefore, selection of cross-resistant HCMV mutants can occur. Thus, there is a need for other therapeutic agents that are safe, potent, and orally bioavailable, with a novel mechanism of action.

As part of an ongoing program to develop novel anti-HCMV compounds that could potentially yield new therapeutic agents for treatment of HCMV, a series of benzimidazole compounds have been evaluated and shown to be potent inhibitors of HCMV replication in vitro (8, 26, 30). One compound, 1H-β-D-ribofuranoside-2-bromo-5,6-dichlorobenzimidazole (BDCRB) (Fig. 1), was shown to be highly selective against HCMV in vitro and relatively nontoxic both to laboratory cell lines and to primary human bone marrow progenitor cells (21, 26). In addition, BDCRB was shown to inhibit HCMV replication by a novel mechanism: unlike GCV, BDCRB did not inhibit HCMV DNA synthesis but instead blocked the maturational cleavage of high-molecular-weight DNA, a step mediated by the products of the UL89 and UL56 genes (17, 27). However, pharmacokinetic studies in rats and monkeys revealed that the glycosidic bond of BDCRB was rapidly cleaved in a first-pass metabolic process, liberating the inactive and significantly more toxic aglycone (2-bromo-5,6-dichloro benzimidazole) (8).

In an attempt to circumvent the metabolic instability of BDCRB, we designed additional compounds that were analogues of BDCRB. We replaced the 2′-halogen with an isopropylamine moiety to create 1H-β-D-ribofuranoside-2-isopropylamino-5,6-dichlorobenzimidazole (3322W93), and we synthesized the biologically unnatural L-sugars corresponding to both BDCRB and 3322W93. The antiviral properties of these compounds were assessed by standard assays. Compared to the parent compound, BDCRB, one of these derivatives,

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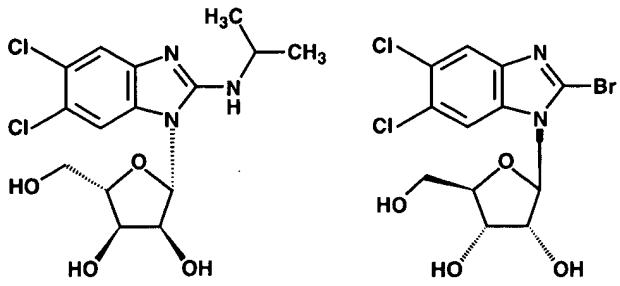
† This paper is dedicated to the memory of Mary Lou Hemphill.



1263W94 BDCRB

FIG. 1. Chemical structures of 1263W94 and BDCRB.

1H-β-L-ribofuranoside-2-isopropylamino-5,6-dichlorobenzimidazole (1263W94; Fig. 1), showed significantly increased potency in vitro, similarly low cytotoxicity, and a unique mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents. BDCRB and its L-ribofuranosyl analog (3858W92) were synthesized according to previously published procedures (26). Compound 1263W94 (Fig. 1) and its D-ribofuranosyl and carbocyclic analogs (3322W93 and 2916W93, respectively) were synthesized according to procedures previously described (4). [^{3}H]-1263W94 (14.9 Ci/mmol) was synthesized by Moravek Biochemicals, Inc. (Brea, Calif.). GCV triphosphate and the 1263W94 5'-mono- and triphosphates were prepared by the previously described chemical method (19). [α-^{32}P]deoxynucleoside triphosphates ([α-^{32}P]dNTPs) were obtained from Amersham (Piscataway, N.J.). Cell media and reagents were obtained from Gibco-BRL (Grand Island, N.Y.), except as noted.

Cell and viral culture. HCMV strain AD169 was obtained from the American Type Culture Collection (Rockville, Md.) and was the laboratory strain used in characterization of the antiviral activity of 1263W94. GCV-resistant recombinants of AD169 were derived from marker rescue experiments with GCV-resistant clinical isolates and wild-type AD169, as reported previously (1, 7, 13, 25). Ten HCMV clinical isolates were obtained from diverse geographical locations within the United States. The HCMV clinical isolates were recovered from immunocompromised patients, including organ transplant patients and human immunodeficiency virus type 1 (HIV-1)-infected patients who had not yet received therapy for HCMV infection. Human T-cell leukemia lines Molt-4, CEM, and CEM CD4$^+$ and human B-cell leukemia line IM9 cells were obtained from the American Type Culture Collection. Human diploid fibroblast cells (MRC-5 lung cells, embryonic kidney cells, and human foreskin cells) were from BioWhittaker (Walkersville, Md.). Human umbilical vein endothelial cells (HUVEC) and coronary artery smooth muscle cells (CASMC) were obtained from Clonetics (San Diego, Calif.). Clinical strains were used if they tested negative for the presence of mycoplasma by using the Gen Probe Mycoplasma Rapid Detection System (Fisher Scientific, Pittsburgh, Pa.).

Monolayer cultures of human diploid fibroblast cells were grown at 37°C in Eagle minimal essential medium (MEM) supplemented with either 8 or 2% fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah), 2 mM L-glutamine, 50 U of penicillin G/ml, and 50 μg of streptomycin sulfate/ml (designated MEM 8-1-1 with 8% FBS or MEM 2-1-1 with 2% FBS). Media for HUVEC and CASMC were obtained from Clonetics and cultured according to recommended protocols.

Determination of antiviral activity. Assessment of antiviral activity and determination of 50% inhibitory concentrations (IC_{50}s) for 1263W94 and other antiviral compounds were made based on three methods of quantitation of virus: (i) measurement of viral plaque formation (plaque reduction assay), (ii) measurement of extracellular infectious virus in a single cycle of viral replication (yield reduction assay), and (iii) measurement of intracellular viral DNA by specific DNA-DNA hybridization after multiple cycles of viral replication. To define the dose-response curves for each antiviral compound assessed, up to eight concentrations of each compound were tested.

Plaque reduction assays were performed with both cell-free virus and cell-associated clinical strains on MRC-5 cells according to standard protocols (24). Plaques were counted by using a binocular microscope at magnifications of ×10 to ×70. IC_{50}s were calculated from the data by using PROBIT, version 79.3 (SAS, Cary, N.C.).

For the virus yield reduction assay, MRC-5 cells were grown to confluence (1.1×10^5 cells/well) in 24-well plates, infected with cell-free virus at a multiplicity of infection (MOI) of ca. 1 and incubated in MEM 2-1-1 at 37°C in the presence or absence of drug. After 72 h, culture supernatants were removed, and limiting dilutions of virus in the culture supernatant were used to infect monolayers of MRC-5 cells. Inoculation media containing residual concentrations of drugs were removed after viral adsorption. Plaques were quantitated as described for the plaque reduction assay. The IC_{50} values were calculated by fitting viral yield as a function of drug concentration to the Hill equation by weighted linear regression (15, 20).

For assessment of antiviral activity by inhibition of viral DNA synthesis of laboratory strain AD169, 0.7×10^4 MRC-5 cells/well were grown for 3 days to confluence ($\sim 2 \times 10^4$ cells/well) in 96-well plates. Medium was removed to leave 20 μl per well, and 150 PFU of AD169 in 25 μl of MEM 2-1-1 was added to each well, for an MOI of ca. 0.008. After a 10-min centrifugation at 1,500 rpm, plates were incubated at 37°C for 90 min to allow viral adsorption to occur, and 180 μl of MEM 2-1-1 containing no drug or containing specified concentrations of antiviral compounds was added to each well. For each antiviral compound investigated, a range of concentrations was chosen to bracket the expected IC_{50} value. Incubation was continued at 37°C for 6 to 8 days until viral controls without drug showed ~100% cytopathic effect. Since nearly 99% of the cells were infected by virus produced during the first round of replication, measurement of viral DNA at the end of the second round of replication provides a measure of the antiviral effects of compounds regardless of their mechanism of action. For assessment of antiviral activity of various anti-HCMV compounds against clinical HCMV isolates in a DNA hybridization assay, cell monolayers were infected with cell-associated virus stocks of each clinical isolate corresponding to ca. 100 infectious centers (24).

In both cases, culture medium was then removed by aspiration, and cells were lysed by the addition of 50 μl of lysis buffer (100 mM Tris-HCl, pH 8; 50 mM EDTA; 0.2% sodium dodecyl sulfate; 0.1 mg of proteinase K/ml)/well and incubation for 1 to 2 h at 56°C. The lysate was extracted with phenol, and viral DNA in the resulting aqueous phase was measured by quantitative DNA hybridization as previously described (27). IC_{50} values were calculated by fitting viral DNA as a function of the drug concentration to the Hill equation as described above.

Selection of drug-resistant virus. Laboratory strain AD169 was serially passaged in increasing concentrations of 2916W93, beginning with 4 μM 2916W93, approximately twice the IC_{50}, and ending with 80 μM, a concentration higher than the IC_{90} value for 2916W93. Virus amplification was accomplished after cultures achieved maximum cytopathic effect by passage of either cell-associated or cell-free supernatant virus, depending upon the extent of virus infection and the integrity of the cell monolayer.

Virus that grew in 80 μM 2916W93 at passage 13 was purified by three cycles of plaque purification in the absence of 2916W93 selective pressure. The resulting virus strain, designated 2916^{r}A, was compared with parent strain AD169 for the ability to replicate in the presence of benzimidazole compounds 1263W94, 2916W93, and BDCRB. GCV was included in the assays for comparison.

Marker rescue experiments. High-molecular-weight DNA was isolated from virus pellets of strain 2916^{r}A, and a cosmid library was constructed and screened for HCMV DNA by hybridization against defined fragments of HCMV as described previously (27). Marker rescue experiments were performed as previously described (27) with the cosmids and subfragments from 2916^{r}A cotransfected with AD169 infectious DNA. After incubation in the absence of drug until maximum cytopathic effect was achieved, 250,000 PFU from each resulting recombinant pool were plated on MRC5 cells and incubated in the presence of 10 μM 1263W94. Incubation was at 34°C, which gave the optimum selective advantage to the resistant mutants. The supernatant virus yield was measured and the cell monolayer was resuspended and used as the inoculum for the second plating.

DNA sequence analysis. The 2.25-kb DNA fragment encompassing the UL97 open reading frame (ORF) was amplified by PCR from genomic HCMV DNA isolated from wild-type strain AD169, the parent strain 2916^{r}A, and the 2916^{r}A-UL97rec virus. The primers used were CPT0 (5'-TCGACGACGCCGTCTA-3') and CPTZ (5'-CAACCGTCACGTTCCGCG-3'). Sequencing reactions of PCR fragments from the UL97 ORF were performed with primers designed from the published sequence of HCMV AD169 (6) and standard protocols with the ABI 377 fluorescent DNA sequencer (PE Biosystems, Foster City, Calif.). Sequencing data were analyzing by using Sequencher DNA analysis software, version 3.0 (GeneCodes Corporation, Ann Arbor, Mich.).

pUL97 enzyme preparation and assay. The baculovirus construct BVGS-TUL97 (14) encoding the pUL97 protein kinase as a glutathione *S*-transferase

TABLE 1. Sensitivity of HCMV strain AD169 to benzimidazole ribosides and GCV in MRC-5 lung fibroblast cells

Test compound			Mean IC_{50} (μM) ± SEM[a] as determined by:		
Name	Benzimidazole 2-substituent	Ribose configuration	DNA hybridization	Plaque reduction	Yield reduction
1263W94	2-Isopropylamino	L	0.12 ± 0.01 (71)	0.54 ± 0.05 (10)	0.06 ± 0.018 (4)
3322W93	2-Isopropylamino	D	2.3 ± 0.5 (3)	7.5 (1)	1.3 ± 0.3
3858U92	2-Bromo	L	0.9 ± 0.2 (5)	1.3 ± 0.7 (3)	0.5 ± 0.06
BDCRB	2-Bromo	D	0.54 ± 0.08 (20)	0.31 ± 0.06 (6)	0.018 ± 0.001
GCV	NA[b]	Acyclic	0.53 ± 0.04 (117)	6.3 ± 0.7 (6)	0.30 ± 0.15 (2)

[a] The number of experiments is shown in parentheses.
[b] NA, not applicable.

fusion protein was generously provided by D. Coen. The protein was produced and purified exactly as described previously (14). The enzyme was assayed as described, except that sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels from autophosphorylation assays were quantitated after exposure to a phosphorimager screen by using ImageQuant software and, to determine histone phosphorylation, the final reaction mixture was mixed with 150 μl of 0.5% phosphoric acid and applied to a phosphocellulose filter (Millipore MAPHNB010). The filter was washed three times with 300 μl of 0.5% phosphoric acid and air dried. Then, 30 μl of Wallac Optiphase Supermix scintillation fluid was added, and the filter was counted in a Wallac Micro Beta scintillation counter. This filter-binding method reported both autophosphorylation and histone phosphorylation.

The pUL97 protein concentration used was 9 nM in the histone phosphorylation assays and 0.5 μM in the autophosphorylation assays. Reaction times and pUL97 concentrations were such that, in the histone phosphorylation assays, only 5% of the total filter-binding signal was due to autophosphorylation.

Effect of 1263W94 on HCMV DNA synthesis and maturation. For these studies MRC-5 cells were seeded in 24-well plates at ~5×10^4 cells/well and grown for 3 days in MEM 8-1-1 to confluence (~1.1×10^5 cells/well). The cells were infected with AD169 in MEM 2-1-1 at an MOI ranging from 1 to 3 and incubated at 37°C for 90 min to allow viral adsorption. The unadsorbed virus was removed and replaced with 1 ml of MEM 2-1-1. To test the effect of compounds on viral DNA synthesis or maturation, 1263W94, BDCRB, or GCV was added to the medium at the concentrations indicated for each experiment.

For experiments examining DNA synthesis, HCMV-infected cells were treated with drug for 3 to 4 days, and viral DNA was isolated at various times and quantitated by DNA hybridization as described above. For experiments examining the effects of 1263W94 and BDCRB on viral DNA maturation, viral DNA was prepared at various times postinfection and subjected to contour-clamped homogenous electric field gel electrophoresis and DNA hybridization analysis of the proportions of monomeric and concatemeric viral DNA as previously described (27).

A novel method was developed to examine whether 1263W94 inhibited maturation of HCMV DNA, the step in DNA replication and processing affected by BDCRB. The "BDCRB wash-out" was a modification of the experiment described above investigating the effect of 1263W94 or BDCRB on viral DNA maturation. Cells were infected at an MOI of 1.0, and 20 μM BDCRB was added after adsorption. Infected cells were incubated for 4 days to allow maximum accumulation of newly synthesized concatemeric viral DNA. Medium containing BDCRB was then removed, and cultures were washed and refed with medium containing the compound to be tested or with drug-free or BDCRB-containing medium as negative and positive controls, respectively. After 24 h of further incubation, the effects of these inhibitors on the processing and packaging of viral DNA were measured. Since the effects of BDCRB are reversible (26, 27), its removal allows the accumulated concatemeric viral DNA to be rapidly processed to unit length genomes and packaged as infectious virus. Supernatant medium was sampled for measurement of the infectious virus titer. Viral DNA was prepared from the cell monolayers, and the proportions of monomeric and concatemeric viral DNA were measured as described above.

Isolation and assay of HCMV DNA polymerase. HCMV DNA polymerase was partially purified from lysed HCMV-infected MRC-5 cells by centrifugation and dialysis, followed by anion-exchange chromatography by using a DEAE cellulose column (16). Assay mixtures and methods were the same as previously described (16). The activity of HCMV DNA polymerase was assessed in the presence of 100 μM concentrations of 1263W94, 1263W94-5′-monophosphate (1263W94-MP), or 1263W94-5′-triphosphate (1263W94-TP). Because benzimidazole ribosides could compete with one or more of the natural dNTPs, assays evaluating 1263W94 and the phosphorylated derivatives were performed in a series of four reactions, with each reaction having three dNTPs present at saturating conditions and the fourth dNTP present in a concentration at or near its K_m.

Anabolism of 1263W94 in human fibroblast cells. Confluent monolayers of MRC-5 cells in 162-cm^2 flasks, with ca. 10^7 cells per flask, were mock infected or infected with 2×10^7 PFU of HCMV AD169. High-pressure liquid chromatography-purified [^{3}H]-labeled 1263W94 (diluted to 1 Ci/mmol) in dimethyl sulfoxide was added to the MEM 2-1-1 at a final concentration of 0.5 μM. At the indicated times after infection the cells were washed and extracted by suspension in 11 ml of ice-cold 80% (vol/vol) acetonitrile in water at −70°C overnight (3). The cells were removed by centrifugation, the supernatant liquid was evaporated to dryness, and the extract was dissolved in water. 1263W94 and its potential anabolites were separated on a Partisil 5 SAX RacII anion-exchange column (Whatman, Inc., Clifton, N.J.) at pH 5.0 with a gradient of KCl. Because 1263W94 was not well resolved from the monophosphate form, the samples were also analyzed on a μBondapak C18 column (Waters Corp., Milford, Mass.) at pH 3.5 with an acetonitrile gradient. In both cases fractions were collected, scintillation fluid was added, and the amount of ^{3}H was counted.

Cytotoxicity assays. Cytotoxicity of 1263W94, BDCRB, and GCV to bone marrow progenitor cells was determined by evaluating growth inhibition (i.e., CFU of granulocytes-macrophages and burst-forming unit-erythroid [see Table 8]), as previously described (11). Cytotoxicity to human leukemia cell lines was determined as previously described (22).

RESULTS

Antiviral activity of benzimidazole compounds. The antiviral activity of 1263W94 against HCMV strain AD169 grown on MRC-5 lung fibroblast cells is compared to those of other related benzimidazole compounds and of GCV in Table 1. In the benzimidazole series, the most potent compound by DNA hybridization was 1263W94, with a mean IC_{50} by DNA hybridization ~4-fold lower than that for GCV. The relative ranking of antiviral potency of the compounds in the series was similar in each of the three antiviral assays employed.

Activity of 1263W94 and GCV against HCMV AD169 was also measured by the yield reduction assay in HUVEC and CASMC. 1263W94 was active at submicromolar concentrations and was ~5-fold more active than GCV. In experiments performed in triplicate, the mean IC_{50} values (± the standard error [SE]) for 1263W94 in CASMC and HUVEC were 0.08 ± 0.03 and <0.01 μM (the lowest concentration tested), respectively, compared to the mean IC_{50} values of 0.52 ± 0.35 and 1.08 ± 0.22 μM for GCV.

To verify that the antiviral activity of 1263W94 was not restricted to the laboratory strain AD169, the antiviral activity of 1263W94 was assessed against 10 clinical isolates of HCMV. All were sensitive to 1263W94, with IC_{50}s obtained in the DNA hybridization assays that ranged from 0.03 to 0.13 μM for 1263W94 and from 0.15 to 1.10 μM for GCV. Similar results were obtained for these 10 clinical strains in the plaque reduc-

TABLE 2. Susceptibility of HCMV clinical isolates to 1263W94 and GCV[a]

Virus strain	Mean IC_{50} (μM) as determined by:			
	DNA hybridization assay		Plaque reduction assay	
	1263W94	GCV	1263W94	GCV
AD169	0.04	0.40	0.97	4.30
C8301	0.04	0.25	0.12	1.10
C8501	0.10	1.00	0.34	1.00
C8910	0.12	0.70	0.19	1.40
C9207	0.03	0.15	0.44	6.70
C8303	0.09	0.80	0.54	0.80
C8302	0.10	0.30	0.16	2.70
C8803	0.13	0.70	0.21	3.40
C8912	0.04	0.50	0.21	2.60
C9003	0.12	1.10	0.56	1.00
C9213	0.04	0.40	0.40	7.00

[a] Assays were performed with HCMV-infected MRC-5 lung fibroblast cells. Values represent the average of two independent determinations. The standard error of the mean was <30% of the value shown in all assays.

tion assays, with IC_{50}s ranging from 0.12 to 0.56 μM for 1263W94 and from 0.80 to 7.00 μM for GCV (Table 2).

Activities of 1263W94, GCV, and BDCRB against GCV- or BDCRB-resistant strains were assessed by using the multicycle DNA hybridization assay. The mutations conferring resistance to GCV had previously been mapped to UL97 (1, 7, 13, 25) and to viral DNA polymerase (2), and the BDCRB-resistant mutations to UL89 in the HCMV genome (27). The strains tested included recombinant strains in which the GCV resistance mutations were recombined into an AD169 background. As shown in Table 3, there were no significant differences in IC_{50}s of 1263W94 among seven of the eight strains tested. An eighth strain, 2916^{r}A, had been selected for resistance to 2916W93 (see below). Notably, these strains that were resistant to BDCRB or to GCV retained sensitivity to 1263W94.

Isolation of drug-resistant virus. In an effort to confirm that 1263W94 inhibits HCMV through a specific antiviral mechanism that targets a viral gene or gene product, we selected HCMV specifically resistant to 1263W94. Strain AD169 was serially passaged in increasing concentrations of 1263W94 or of the closely related carbocyclic analog 2916W93. The selective pressure resulted in the enrichment of several viral populations capable of variable growth in the presence of the drugs. Plaque purification of the most growth-competent strains selected with 2916W93 yielded strain 2916^{r}A, which clearly showed a drug-resistant phenotype. Cross-resistance was tested by DNA hybridization assays, which showed strain 2916^{r}A to be sensitive to both BDCRB and GCV (Table 3). This finding confirms results presented in Gudmundsson et al. (12). Both the BDCRB-resistant strain 1038^r and the GCV-resistant XbaF4 were sensitive to 1263W94.

A cosmid library was constructed from strain 2916^{r}A, and a fragment identified as S7 was shown to confer resistance to 1263W94 in marker transfer experiments. Analysis of fragment S7 showed that it contained *Hin*dIII fragments P, R, S, and T (6), and resistance to 1263W94 was associated only with *Hin*dIII fragment S (Table 4). At the same time that these results were obtained, we found that 1263W94 was a potent inhibitor of the HCMV UL97 protein kinase (see below), which is encoded by one of the ORFs on *Hin*dIII fragment S. Consequently, the genetic mapping then focused on the UL97 ORF. The 2.25-kb PCR fragment designated PCR-UL97-2916^{r}A encompassing the UL97 ORF and defined by primers CPTZ and CPTO (see Materials and Methods) was recombined with wild-type strain AD169, and the recombinant progeny virus was found to be resistant to 1263W94 (Table 4). DNA sequencing of the 2.25-kb fragment identified a single nucleotide substitution, T1190G, which would result in a predicted amino acid substitution of Leu to Arg at position 397 of the UL97 polypeptide.

After plaque purification the recombinant virus, now designated 2916^{r}A-UL97rec, was compared to its parents, the wild-type AD169 and the 2916^{r}A viruses, in a multicycle DNA hybridization assay for resistance phenotype. The IC_{50}s for 1263W94 were 0.056 ± 0.01 μM for AD169, 0.7 ± 0.3 μM for 2916^{r}A, and 0.9 ± 0.02 μM for 2916^{r}A-UL97rec. These data suggest that the mutation in UL97 is sufficient to account for the observed resistance of 2916^{r}A to 1263W94.

1263W94 inhibits wild-type UL97 protein kinase but not UL97 kinase from the 2916^{r}A virus. 1263W94 was a potent inhibitor of histone phosphorylation catalyzed by wild-type

TABLE 3. Sensitivity of drug-resistant strains of HCMV to benzimidazole ribosides and GCV, as determined by a DNA hybridization assay

Virus strain	Mean IC_{50} ± SEM (μM)[a]			Altered gene	Amino acid change (s)
	1263W94	GCV	BDCRB		
AD169	0.08 ± 0.02	0.31 ± 0.1	0.7 ± 0.3	NA[b]	NA
8805^{rec}1	0.03 ± 0.01	1.7 ± 0.1	2 ± 1	UL97	Mct460Val
9330^{rec}5	0.13 ± 0.06	1.6 ± 0.4	0.7 ± 0.3	UL97	His520Glu
8702^{rec}2	ND[d]	1.8 ± 0.3	0.75 ± 0.3	UL97	Ala594Val
9219^{rec}7	0.2 ± 0.1	1.8 ± 0.3	1 ± 0.8	UL97	Leu595del
XbaF.4	0.03 ± 0.01	4.1 ± 0.9	0.2 ± 0.2	UL97	AlaAlaCysArg593del
4955^{rec}	0.06 ± 0.03	0.7 ± 0.07	ND	pol[c]	Thr700Ala
1038^{r}B	0.04 ± 0.02	0.24 ± 0.06	>20	UL89	Asp344Glu and Ala355Val
2916^{r}A[e]	2.7 ± 0.6	0.31 ± 0.04	0.48 ± 0.12	UL97	Leu397Arg

[a] That is, the mean values based on a minimum of three independent determinations.
[b] NA, not applicable.
[c] pol, polymerase.
[d] ND, not determined.
[e] DNA sequencing of the entire virus was not performed. The sequence change in the UL97 ORF conferred resistance to 1263W94 when transferred into wild-type strain AD169.

TABLE 4. Virus yields in the presence of 10 μM 1263W94 after recombinant pools from marker rescue of HCMV AD169 were plated

DNA fragment from 2916^{r}A recombined with AD169	Virus yield (PFU/ml)	
	Plating 1	Plating 2
None	100	300
PCR-UL97-2916^{r}A	60	212,300
S7	10,000	37,500
S7 8.5-kb *Hin*dIII	2,400	17,500
S7 *Hin*dIII-P	120	1,600
S7 *Hin*dIII-R	80	1,000
S7 *Hin*dIII-S	6,500	2,220,000
S7 *Hin*dIII-T	120	11,500

pUL97 in vitro, with an IC_{50} of 3 nM, close to one-half of the concentration of active enzyme. In contrast, the IC_{50} of 1263W94 for histone phosphorylation catalyzed by the UL97 kinase with the Leu397Arg mutation of the 2916^{r}A virus was increased 20,000-fold to 60 μM. IC_{50} determinations were not performed for autophosphorylation; however, wild-type pUL97 autophosphorylation was inhibited 99% by 1 μM 1263W94. Autophosphorylation of pUL97 from the 2916^{r}A virus was inhibited by <5% by 1 μM 1263W94.

1263W94 inhibits HCMV DNA synthesis in infected cells but does not directly inhibit HCMV DNA polymerase. Viral DNA was measured in HCMV-infected MRC-5 cells during a single-cycle infection in the presence or absence of the antiviral drugs BDCRB, 1263W94, or GCV (Fig. 2). Synthesis of HCMV DNA was unaffected by the presence of 1.5 μM BDCRB, as previously reported (27). However, the continuous presence of 1 μM 1263W94 or GCV, viral DNA synthesis was reduced to <10% of control levels. These measurements were repeated with a range of concentrations of 1263W94 from 0.025 to 0.5 μM and of GCV from 0.5 to 5 μM, permitting the calculation of IC_{50}s for inhibition of DNA synthesis by 1263W94 and GCV at different times postinfection. At 72 h postinfection the IC_{50} was 0.19 ± 0.07 μM for 1263W94 and 1.9 ± 0.3 μM for GCV; at 92 h postinfection the IC_{50} was 0.11 ± 0.01 μM for 1263W94 and 0.8 ± 0.13 μM for GCV. The

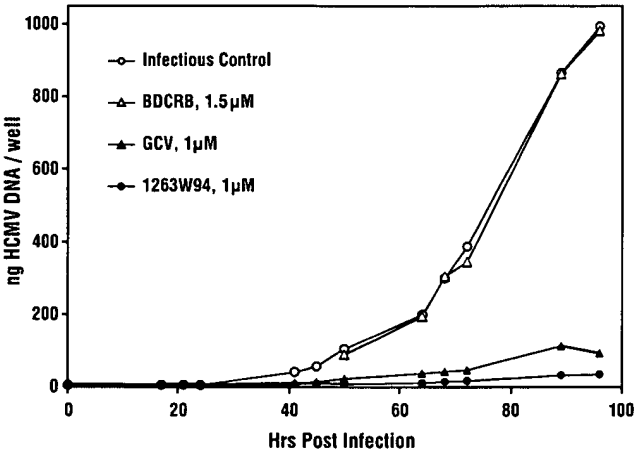


FIG. 2. Effects of 1263W94, GCV, and BDCRB on HCMV DNA synthesis during a single-cycle infection of MRC-5 cells.

TABLE 5. Relative inhibition at 72 h postinfection of virus DNA synthesis and virus yield by 1263W94 and other compounds in a single-cycle infection

Test compound	Mean IC_{50} ± SEM (μM)[a]	
	DNA synthesis	Virus yield
1263W94	0.28 ± 0.03	0.06 ± 0.018
BDCRB	>25	0.03 ± 0.005
GCV	0.95 ± 0.1	0.3 ± 0.15
PFA	79 ± 11	33 ± 1

[a] That is, of at least three determinations.

IC_{50}s for inhibition of HCMV DNA synthesis by 1263W94 and GCV in a single cycle of viral replication are similar to those measured for viral replication by any of the standard methods employed. These observations were extended by comparing the inhibition by 1263W94, BDCRB, GCV, and phosphonoformic acid (PFA) (10) of HCMV DNA synthesis and of viral yield at 72 h postinfection (Table 5). For GCV and PFA, the IC_{50}s for DNA synthesis were two- to threefold higher than the IC_{50}s for yield reduction. Similarly, for 1263W94 the IC_{50}s for DNA synthesis were four- to fivefold higher than those for yield reduction; in contrast, for BDCRB the IC_{50}s for DNA synthesis were >750-fold higher than those for yield reduction.

To investigate whether inhibition of viral DNA synthesis by 1263W94 resulted from a direct effect on viral DNA polymerase, HCMV DNA polymerase activity was assessed in the presence of 1263W94, 1263W94-MP, or 1263W94-TP. No significant inhibition of HCMV DNA polymerase was observed in the presence of 100 μM concentrations of any of the compounds tested (Table 6). GCV triphosphate inhibited this HCMV polymerase preparation with a K_i value of 0.43 μM. Thus, 1263W94 inhibits viral DNA synthesis but by a mechanism different from that of GCV.

Phosphorylation of 1263W94 to 1263W94-MP or 1263W94-TP does not occur in uninfected or HCMV-infected human fibroblasts. To determine whether human fibroblast cells produce phosphorylated anabolites of 1263W94, both uninfected and HCMV-infected MRC-5 cells were cultured in the presence of radiolabeled 1263W94. Anion-exchange and reversed-phase high-pressure liquid chromatography were used to examine cell extracts prepared at 0, 48, and 96 h postinfection. The sensitivities of the methods employed were such that intracellular concentrations of as little as 10 nM would have been detected. No phosphorylated anabolites of 1263W94 were observed in either uninfected or HCMV-infected cells (data not shown).

Effect of 1263W94 on HCMV DNA maturation. Previous reports demonstrated that BDCRB blocks HCMV replication

TABLE 6. Effect of 1263W94 and phosphorylated derivatives on the activity of HCMV DNA polymerase

Inhibitor	HCMV DNA polymerase (% activity by limiting dNTP)			
	dATP	dCTP	dGTP	TTP
1263W94	91	96	90	91
1263W94-MP	84	96	101	102
1263W94-TP	86	89	100	108

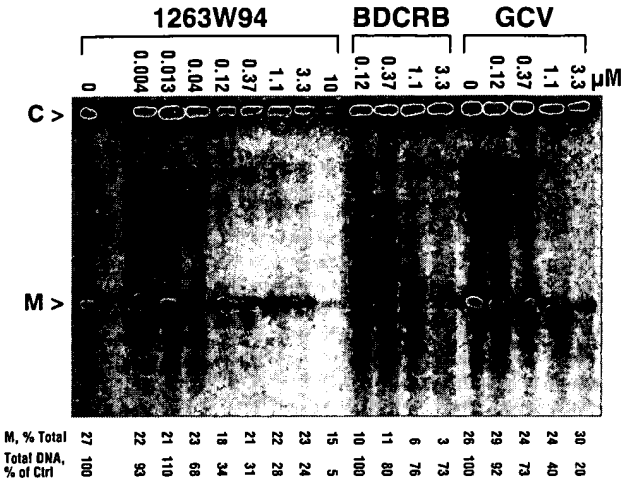


FIG. 3. Synthesis and processing of high-molecular-weight HCMV DNA in HCMV-infected cells in the presence or absence of 1263W94, BDCRB, and GCV. Calculations from integrated band intensities are shown below the figure. Abbreviations: M, monomeric viral DNA; C, concatemeric viral DNA.

by inhibiting the maturational cleavage of high-molecular-weight viral DNA concatemers (27), an obligatory process, conserved in all herpesviruses, that occurs in conjunction with viral DNA packaging (28). To determine whether 1263W94 inhibits this process, the accumulation of high-molecular-weight viral DNA in HCMV-infected cells was measured in the presence or absence of 1263W94 (Fig. 3). The total amount of HCMV DNA synthesized decreased with increasing concentrations of 1263W94, but there were only minor changes in the extent of conversion of the precursor HCMV concatemers to mature unit-length viral DNA. In contrast, BDCRB did not inhibit the total amount of HCMV DNA synthesized but

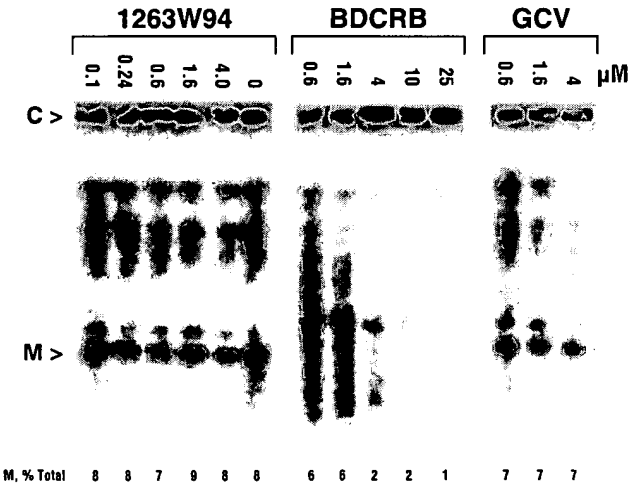


FIG. 4. "BDCRB washout" experiment for determining the effects of 1263W94, BDCRB, and GCV on the processing of concatemeric HCMV DNA that had accumulated during 96 h in the presence of BDCRB. Calculations from integrated band intensities are shown below the figure. Abbreviations: M, monomeric viral DNA; C, concatemeric viral DNA.

TABLE 7. Effect of drug treatment on HCMV DNA maturation and virus yield after reversal of BDCRB treatment

Test compound	IC_{50} ± SEM (μM)	
	DNA maturation	Virus yield
1263W94	27 ± 7	18 ± 0.5
BDCRB	0.1 ± 0.1	0.08 ± 0.01
GCV	>25	>25

clearly inhibited the conversion of HCMV concatemers to monomers in a dose-dependent manner.

The "BDCRB washout" method was also employed to examine more directly the effects of 1263W94 on DNA concatemer cleavage. As shown in Fig. 4 and as quantified in Table 7, the exposure of infected cells to 1263W94 only after removal of the BDCRB-mediated block in viral maturation did not prevent subsequent production of mature HCMV DNA and infectious virus. BDCRB inhibited both measures of viral maturation, with IC_{50} calculated to be 0.1 μM.

Cytotoxicity results. Like other benzimidazoles (21), 1263W94 was significantly less toxic to human bone marrow progenitor cells than was GCV (Table 8). Growth inhibition studies with 1263W94 were also conducted in three human T-cell leukemia lines (Molt-4, CEM, and CEM-$CD4^+$) and one human B-cell leukemia line (IM9). The IC_{50}s ranged from 35 μM for MOLT-4 cells to 72 μM for CEM-$CD4^+$ cells (data not shown).

DISCUSSION

The benzimidazole ribosides are a series of compounds with potent antiviral activity against HCMV. The lead compound in the series, BDCRB, proved to be disappointing due to its rapid metabolism to the inactive and toxic aglycone (8). In an effort to discover metabolically stable derivatives with the anti-HCMV activity of BDCRB, various D- and L-riboside analogs of BDCRB were synthesized. Synthesis and analysis of a number of riboside derivatives in the benzimidazole series has led to a preliminary description of the structure activity relationship of the series. The compounds described here represent the 2′-substituted D- and L-riboside analogs that were the most thoroughly characterized.

In the benzimidazole series tested, both the biologically unnatural L-riboside derivatives and the D-riboside derivatives were potent antiviral agents against laboratory HCMV strains in vitro. The L-riboside of BDCRB, 3858W92, showed an anti-HCMV activity but was less potent than BDCRB (Table 1).

TABLE 8. Cytotoxicity of benzimidazole ribosides and GCV to primary human bone marrow progenitor cells[a]

Test compound	Mean IC_{50} (μM) ± SEM		
	CFU-GM	BFU-E	No. of determinations (no. of donors)
1263W94	90 ± 4	88 ± 4	4 (4)
BDCRB	110 ± 18	90 ± 10	9 (9)
GCV	15 ± 3	39 ± 10	18 (15)

[a] Data for 1263W94 were previously reported by Chan et al. (5). CFU-GM, CFU of granulocytes-macrophages; BFU-E, burst-forming unit-erythroid.

Substitution of a 2′-isopropyl moiety for the 2′-bromo moiety of BDCRB and 3858W92 produced the 2′-isopropylamino D-riboside analog 3322W93 and the corresponding L-riboside 1263W94. The D-riboside 3322W93 was somewhat less potent than BDCRB. In contrast, 1263W94 exhibited an anti-HCMV potency comparable to that of BDCRB in each of the viral assays performed, with IC_{50}s 4- to 10-fold lower than those of GCV. Furthermore, 1263W94 was highly active against GCV- or PFA-resistant HCMV strains and against a panel of HCMV clinical isolates from different geographical areas of the United States.

The D-riboside compounds in the benzimidazole series, BDCRB and 3322W93, do not inhibit viral DNA synthesis but instead inhibit viral replication at the stage of viral DNA maturation, when the viral DNA concatemers are processed to form unit length DNA for viral packaging (27). An unexpected finding in our studies was that the antiviral activity of 1263W94 stems from a different mechanism of action from that of BDCRB, the parent compound. As shown by direct analysis (Fig. 3) and by the "BDCRB washout" experiment (Fig. 4), incubation of HCMV-infected cells in the presence of increasing concentrations of 1263W94 does not significantly inhibit processing of concatemeric DNA. As shown in Fig. 2 and Table 5, 1263W94 and GCV both inhibit viral DNA synthesis in a single cycle assay, and the ratios of the IC_{50}s for DNA synthesis and viral yield are similar for both compounds. Like GCV, 1263W94 inhibits viral DNA replication, but it does so by a different mechanism of action. This conclusion is consistent with the lack of cross-resistance of the 1263W94- and GCV-resistant viral strains examined in this study.

The antiviral activity of GCV results from incorporation of the triphosphorylated metabolite of GCV into the growing DNA chain as GCV monophosphate (18). The mechanisms of antiviral action of the benzimidazole compounds differ from that of GCV, since they are active against viral strains resistant to GCV. Unlike GCV (18), 1263W94 is not phosphorylated in infected cells, nor does the compound itself nor any of its synthetic phosphorylated derivatives inhibit HCMV DNA polymerase. The antiviral activity of 1263W94 results from a novel mechanism of action, one different from that of both GCV and the parent compound, BDCRB.

We show here that resistance to 1263W94 in the HCMV mutant 2916^{r}A maps to the UL97 ORF and that 1263W94 is a potent inhibitor of the UL97 protein kinase of HCMV. Wolf et al. (29) have recently reported that a UL97 deletion mutant of HCMV shows a reduction in DNA synthesis comparable to that described here in the presence of 1263W94. It is probable, therefore, that the primary target of 1263W94 is the UL97 kinase, but as yet it is not known how inhibition of the kinase results in inhibition of viral replication.

Wolf et al. also presented the results of BDCRB washout experiments showing that the UL97 deletion mutant was defective in capsid assembly, a finding inconsistent with those presented here (29). Since deletion and inhibition of an enzyme may not be exactly equivalent, the inconsistency may be more apparent than real. In any case, our data cannot exclude the possibility that pUL97 is also involved in late stages of viral replication. Indeed, data presented in Fig. 3 indicate that 1263W94 at higher concentrations reduces the formation of monomeric DNA. The lack of measurable effect of 1263W94 on DNA concatemer maturation in the BDCRB washout experiments (Fig. 4) could indicate that the effects of 1263W94 on monomer formation, presumably mediated through inhibition of pUL97, occur earlier in replication.

Others have observed less-potent inhibition of HCMV DNA replication by 1263W94 than shown here (D. Coen, unpublished data). Such differences in experimental observations on the role of pUL97 in viral replication could result from different experimental conditions, particularly in the physiological state of the infected cells resulting from differences in culture conditions. All of the experiments reported here were carried out with fully confluent, quiescent cells, conditions that yielded the most reproducible replication of HCMV strain AD169.

In vitro assays indicate that 1263W94 shows not only a potency advantage over GCV but also a selectivity advantage. One of the side effects associated with prolonged GCV therapy is bone marrow toxicity, resulting in leukopenia, thrombocytopenia, anemia, and bone marrow hypoplasia (9). Previous work had shown BDCRB and certain related benzimidazole nucleosides to be less toxic to bone marrow cells in vitro than GCV (21). Our data with 1263W94 extended these results, showing that both benzimidazole compounds have low cytotoxicity, reducing the possibility of bone marrow suppression with therapy.

In conclusion, we have shown that 1263W94 is a novel, selective, and potent anti-HCMV compound with a novel mechanism of action. The mechanism has proven relevant to antiviral therapy, since 1263W94 (maribavir) has recently shown potent reduction of viral load in a phase I-II clinical study in HIV-infected patients (J. Lalezari, J. Aberg, L. Wang, M. Wire, R. Miner, W. Snowden, C. Talarico, S. Shaw, M. Jacobson, and W. Drew, unpublished data).

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Phase I Dose Escalation Trial Evaluating the Pharmacokinetics, Anti-Human Cytomegalovirus (HCMV) Activity, and Safety of 1263W94 in Human Immunodeficiency Virus-Infected Men with Asymptomatic HCMV Shedding

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1263W94 [maribavir; 5,6-dichloro-2-(isopropylamino)-1,β-L-ribofuranosyl-1-H-benzimidazole] is a novel benzimidazole compound for treatment of human cytomegalovirus (HCMV) infection and disease, with potent in vitro activity against HCMV and good oral bioavailability. A phase I study was conducted to determine the pharmacokinetics (PK), anti-HCMV activity, and safety of 1263W94 administered as multiple oral doses to human immunodeficiency virus type 1-infected adult male subjects with asymptomatic HCMV shedding. Subjects received one of six dosage regimens (100, 200, or 400 mg three times a day, or 600, 900, or 1,200 mg twice a day) or a placebo for 28 days. 1263W94 demonstrated linear PK, with steady-state plasma 1263W94 profiles predictable based on single-dose data. 1263W94 was rapidly absorbed following oral dosing, and values for the maximum concentration of the drug in plasma and the area under the concentration-time curve increased in proportion to the dose. 1263W94 demonstrated in vivo anti-HCMV activity in semen at all of the dosage regimens tested, with mean reductions in semen HCMV titers of 2.9 to 3.7 $\log_{10}$ PFU/ml among the four regimens evaluated for anti-HCMV activity. 1263W94 was generally well tolerated; taste disturbance was the most frequently reported adverse event over the 28-day dosing period.

Infection with human cytomegalovirus (HCMV) is common, with seroprevalence ranging from approximately 50 to 60% of adults in Western Europe and the United States to as much as 100% of some adult populations (6, 13). In immunocompetent HCMV-infected individuals, the virus normally remains latent and does not constitute a major health risk. However, HCMV infection can be a serious complication in immunologically immature individuals, such as neonates, or in immunocompromised individuals, such as solid-organ transplant recipients, bone marrow transplant recipients, or people with AIDS.

There are six currently approved therapies in the United States for treatment or prevention of systemic HCMV infection or HCMV retinitis associated with AIDS (6, 13). Treatment of systemic HCMV infection requires administration of ganciclovir, foscarnet, or cidofovir. After induction therapy with intravenously (i.v.) administered agents or oral valganciclovir, maintenance therapy can be provided by oral ganciclovir or valganciclovir. An intravitreal implant of ganciclovir is available for treatment of HCMV retinitis; however, the implant is insufficient for controlling systemic disease and must be used together with systemic therapy, such as oral or i.v. ganciclovir or oral valganciclovir. Fomivirsen, administered by intravitreal injection, is approved as a second-line therapy for treatment of HCMV retinitis in subjects who cannot tolerate or fail to respond to first-line therapy. Disadvantages of the currently approved therapies include treatment-limiting toxicities, such as bone marrow suppression (ganciclovir) and nephrotoxicity (foscarnet and cidofovir), limited penetration to target sites, and inconvenient i.v. dosing or poor oral bioavailability (oral ganciclovir) (13). Thus, there is a need for a safe and effective oral therapy for the treatment and prevention of HCMV disease.

1263W94 [maribavir; 5,6-dichloro-2-(isopropylamino)-1,β-L-ribofuranosyl-1-H-benzimidazole] is a novel benzimidazole compound (3) shown to have antiviral activity against HCMV in vitro (1). 1263W94 does not require intracellular activation and has demonstrated activity against clinical isolates resistant to ganciclovir or foscarnet (1).

1263W94 was safely administered as single oral doses of 50 to 1,600 mg to healthy and human immunodeficiency virus (HIV)-infected adults in two separate studies (L. Wang, L., R. Peck, Y. Yin, J. Allanson, R. Wiggs, and M. Wire, unpublished data). In the two single oral dose escalation studies, 1263W94 pharmacokinetics (PK) were dose proportional over the dose range tested, 1263W94 was highly metabolized (~40% of the dose was recovered in the urine as metabolite, and <2% was recovered as the parent drug), and 1263W94 was highly

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(~98.5%) protein bound. Previous in vitro human liver microsomal studies suggested that CYP3A4 was the primary enzyme responsible for 1263W94 metabolism (N dealkylation) (Wang et al., unpublished). Indeed, ~40% of the 1263W94 dose recovered in the urine was recovered as the N-dealkylated metabolite (Wang et al., unpublished).

We conducted a phase I dose escalation study to evaluate the PK, anti-HCMV activity, and safety of 1263W94 in HIV-1-infected adult males with asymptomatic HCMV shedding in urine and semen. Quantitative reductions in semen HCMV concentrations are dose responsive and predictive of clinical efficacy (10–12).

MATERIALS AND METHODS

Study population and investigative sites. The study population consisted of HIV-1-infected males. Eligibility criteria were as follows: all subjects had to be ≥18 years old, have a life expectancy of >6 months, and be stable on all chronically administered therapy for HIV infection and opportunistic infections for at least 1 month. Subjects were stratified into two groups, main and satellite, on the basis of semen and urine HCMV culture results prior to study entry. Subjects with a semen HCMV concentration of ≥5,000 PFU/ml and with an HCMV-positive urine culture within 30 days of study entry (day 1) were enrolled in the main group for evaluation of the PK, anti-HCMV activity, and safety of 1263W94. There was no requirement for detectable HCMV infection in subjects enrolled in the satellite group for evaluation of 1263W94 PK and safety. Subjects enrolled in the satellite group were required to have a $CD4^+$ cell count of <150 cells/mm^3 or <10% of total lymphocytes. For subjects with a $CD4^+$ cell count of <100 cells/mm^3 or with signs or symptoms of HCMV disease, an indirect fundoscopic examination was performed by an ophthalmologist to rule out HCMV retinitis. The following exclusion criteria applied to all subjects: active HCMV disease or history of HCMV disease; visual symptoms suggestive of HCMV disease unless HCMV disease was excluded by ophthalmoscopic examination; treatment with ganciclovir, foscarnet, cidofovir, or investigational anti-HCMV drugs within 2 months prior to study entry (day 1); treatment with interferons, immunomodulatory agents, or HCMV hyperimmune globulin within 1 month prior to study entry (day 1); active hepatitis, obstructive hepatobiliary disease, or cirrhosis; gastrointestinal disorders that could interfere with oral dosing or drug absorption or that might indicate HCMV disease; known history of lactose intolerance; diagnosis of chronic diseases that could compromise the safety or compliance of the subject; treatment with radiation therapy or systemic therapy for visceral malignancy within 2 months prior to study entry (day 1), or anticipated need for such treatment during the study period; participation in an investigational trial or treatment with an investigational therapy within 2 months (anti-HCMV therapy) or 1 month (other therapy) prior to study entry (day 1); abnormal laboratory values within 14 days of study entry (day 1), notably, hemoglobin of <8.5 g/dl, a neutrophil count of <750 cells/mm^3, a platelet count of ≤50,000 cells/mm^3, AST, ALT, or alkaline phosphatase levels >4 times the upper limit of normal, total bilirubin of >2 mg/dl, or estimated creatinine clearance of <50 ml/min; or a debilitated condition resulting from HIV disease or associated illnesses or therapies such that the subject was considered unable to complete the study.

The study was conducted from 27 August 1996 through 9 July 1997 at three sites in San Francisco, Calif. Quest Clinical Research, University of California San Francisco (UCSF) Mount Zion Medical Center, and San Francisco General Hospital were the three participating centers. The study was approved by the Western Institutional Review Board (Olympia, Wash.) and by the UCSF Committee on Human Research (San Francisco, Calif.) and was conducted under Good Clinical Practices guidelines. All subjects provided written informed consent before any study procedures were performed.

The Virology Research Laboratory at the UCSF Mount Zion Medical Center performed the plaque assays and processed samples for shipment to GlaxoWellcome for HCMV DNA PCR analysis, viral sensitivity testing, and measurement of 1263W94 concentrations.

Study design. The study was a phase I multiple-dose, randomized, parallel dose escalation study. Eligible subjects were stratified to the main group or the satellite group on the basis of quantitative HCMV culture in semen and qualitative HCMV culture in urine, as described above. The study was designed to evaluate the PK and safety of 1263W94 in both groups and the anti-HCMV activity of 1263W94 in the main group.

Subjects in the main group received open-label 1263W94 at one of the following dosage regimens: 100, 200, or 400 mg three times a day (t.i.d.) or 600 mg twice a day (b.i.d.). Subjects in the satellite group were randomized in a double-blind fashion to receive 1263W94 at a particular dosage regimen (100, 200, or 400 mg t.i.d. or 600, 900, or 1,200 mg b.i.d.) or a matching placebo. Subjects were sequentially enrolled into the escalating-dose cohorts. The study was designed to include a prescreening visit on day −30, a screening visit at day −14, enrollment and initial dosing on day 1, weekly visits on days 7, 14, 21, and 28, and a follow-up visit approximately 4 weeks after the final dosing on day 28 (i.e., day 56). Prescreening assessments of HCMV in semen and urine were performed to determine whether to assign subjects to the main group or the satellite group, as described above. Screening assessments included a test for HIV-1 antibody (enzyme-linked immunosorbent assay), complete medical history, review of inclusion-exclusion criteria, demographic data, ocular examination (if necessary), and clinical evaluations (including physical examination, vital signs, height and weight measurements, assessment of HIV-related conditions, assessment of concurrent medications, and clinical laboratory evaluations, including hematology, $CD4^+$ lymphocyte count, serum chemistry, and urinalysis). On-study assessments included clinical evaluations as described above, review of adverse events, PK evaluations on specified visit days, and HCMV assessments on specified visit days.

The study drug was administered on an outpatient basis, without regard to meals, except on days 1 and 28, when serial PK samples were collected following an overnight fast (at least 8 h). A single dose of the study drug was given on day 1, and routine b.i.d. or t.i.d. dosing began on day 2.

PK sampling. Serial plasma samples were collected on days 1 and 28. Subjects were required to fast the evening before dosing on days 1 and 28 and not to take the morning dose (day 28) before arriving at the clinic. Each subject had a cannula inserted into a suitable forearm vein before dosing. A predose blood sample was collected, and the dose was given orally with 480 ml of water. Subjects were required to fast for another 3 h after receiving the dose; afterwards, a regular lunch and dinner were provided at the clinic. Seven-milliliter whole-blood samples were collected in tubes containing EDTA at the following times after dosing: 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, and 24.0 h. Each sample was immediately refrigerated or stored on ice until centrifugation. Within an hour after collection, blood was separated by centrifugation, and plasma was aliquoted into two polypropylene storage tubes. Plasma samples were stored upright at −20°C.

All subjects collected semen samples to bring to the clinic on days 1, 14, and 28; subjects enrolled in the main group collected additional semen samples for the clinic visits on days 7 and 21. Following protocol amendments, subjects in the main group of the 400-mg t.i.d. and 600-mg b.i.d. cohorts also collected semen samples for the day-4 visit. Samples were collected at home in sterile containers, either on the evening before the day of the clinic visit or on the morning of the visit day. The timing of the semen collection was not standardized relative to the time of dosing or relative to the time of plasma PK sampling. Samples collected in the evening were stored in the refrigerator overnight. A 50- to 100-μl semen aliquot was transferred to a polypropylene storage tube and stored at −20°C until shipment to GlaxoWellcome for measurement of 1263W94 concentrations by a validated assay.

On days 1 and 28, subjects were asked to void before dosing, and urine samples for PK analysis were collected at 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h postdosing. Urine samples were stored in the refrigerator over the collection interval. A 10-ml aliquot from each urine collection interval was transferred to a polypropylene storage tube, and samples were stored upright at −20°C until shipment to GlaxoWellcome for measurement of 1263W94 concentrations by a validated assay.

Bioanalysis of PK samples. Plasma, urine, and semen samples were analyzed for 1263W94 by use of separately validated high-performance liquid chromatography-mass spectrometry (MS)-MS methods following solid-phase extraction. For plasma, the validated calibration range was 50 to 6,000 ng/ml, the accuracy (expressed as percent bias) was ±5.1%, and the global precision (expressed as the coefficient of variation [CV]) was 14.1%. For urine, the validated calibration range was 50 to 50,000 ng/ml, the accuracy (percent bias) was ±9.4%, and the global precision (CV) was 10.0%. For semen, the validated calibration range was 50 to 7,500 ng/ml, the accuracy (percent bias) was ±10.5%, and the global precision (CV) was 10.2%.

Plasma PK analysis. PK analyses of plasma 1263W94 concentration-versus-time data obtained following single-dose administration on day 1 and following multiple dosing to steady state on day 28 were conducted by noncompartmental methods, with the log-linear trapezoidal option in WinNonlin Professional, version 1.5 (Pharsight Corporation, Mountain View, Calif.). Values were estimated for the maximum concentration of 1263W94 in plasma (C_{max}) and C_{max} at steady state ($C_{max,ss}$), time to maximum concentration (T_{max}) and T_{max} at steady state ($T_{max,ss}$), minimum concentration during a dosing interval at steady state

($C_{min,ss}$), apparent terminal elimination half-life ($t_{1/2}$), area under the concentration-time curve (AUC_∞ and $AUC_{\tau,ss}$), and average concentration at steady state ($C_{avg,ss}$). The daily area under the concentration-time curve at steady state ($AUC_{24,ss}$) was calculated as $AUC_{\tau,ss} \cdot 24\ h/\tau$.

Urine PK analysis. Analyses of urinary excretion data for 1263W94 were performed to determine the percentage of the dose recovered as 1263W94. The urine 1263W94 concentration was multiplied by the volume of urine collected during each urine collection interval, divided by the dose of drug administered, and multiplied by 100. The cumulative percentage of the dose excreted in urine as 1263W94 was determined by summing the percentages of the dose excreted in each urine collection interval.

Statistical analysis of 1263W94 PK. Descriptive statistics were calculated for plasma and urine 1263W94 PK parameters. Log-transformed AUC_∞ and $AUC_{\tau,ss}$ were compared within-subject by using analysis of variance (SAS, version 6.08, MIXED procedure [SAS Institute, Cary, N.C.]) to assess the time invariance of 1263W94 PK. The geometric least-squares mean (LSM) for each PK parameter was determined, and the ratio of the geometric LSM for $AUC_{\tau,ss}$ to that for AUC_∞ was calculated, along with the associated 90% confidence intervals (90% CI).

Dose proportionality was assessed by fitting the data to a power model, relating log-transformed C_{max}, AUC_∞, and $AUC_{24,ss}$ to the log-transformed dose (log-transformed parameter = $\alpha + \beta \cdot$log-transformed dose), by restricted maximum likelihood using the MIXED procedure. The common slope was estimated, and the associated 90% CI was constructed to examine linearity. The proximity to unity of the slope estimate for AUC_∞ was considered the primary assessment of dose proportionality, and the proximity to unity of the slope estimate for C_{max} was considered the secondary assessment of dose proportionality.

HCMV sampling. Stratification of subjects to the main or satellite group was determined by prescreening HCMV assessments that consisted of quantitation of HCMV in semen by using a plaque assay and a qualitative analysis of the presence of HCMV in urine. HCMV assessments for main-group subjects during the study included the following: quantitative evaluation of HCMV in semen (days 1, 4, 7, 14, 21, and 28) by both plaque titration and a PCR-based assay, quantitative evaluation of HCMV DNA in whole blood (days 1, 4, 7, 14, 21, and 28) by a PCR-based assay, qualitative HCMV culture from urine (days 1, 4, 7, 14, 21, and 28), and isolation of HCMV from semen and urine for assessment of the sensitivity of the virus to 1263W94 (days 1, 28, and 56). All samples for HCMV testing were collected prior to dosing on the visit day.

Semen samples were collected as described for PK sampling above. Immediately after collection, semen samples were processed by the Virology Research Laboratory for subsequent analyses. Semen lysates containing cell-free virus were obtained for quantitative PCR-based analysis of HCMV concentrations by diluting an aliquot of the semen sample 1:10 in minimal essential medium (MEM; Gibco BRL, Gaithersburg, Md.), lysing the cells by sonication, and filtering the lysate. A 0.5-ml aliquot of the filtered semen lysate was transferred to a polypropylene storage tube and stored upright at −70°C until shipment to GlaxoWellcome.

Whole-blood samples for quantitation of HCMV by a PCR-based assay were collected in 4-ml EDTA-containing tubes. These samples were stored upright at −70°C until shipment to GlaxoWellcome for analysis. For qualitative urine HCMV culture, 10- to 20-ml aliquots from each urine collection were refrigerated and later processed by the Virology Research Laboratory.

Cell culture and media. Laboratory strain AD169 (American Type Culture Collection, Manassas, Va.) was used as the wild-type HCMV reference strain, and strain 2916^r was used as a reference 1263W94-resistant strain. 2916^r is a derivative of AD169 selected for growth in the presence of the benzimidazole compound 2916W93 (1, 7). Human foreskin fibroblasts (HFF) and MRC-5 human lung fibroblasts were obtained from BioWhittaker (Walkersville, Md.) and used between passages 20 and 30.

Except as noted, reagents and cell media were obtained from Gibco BRL. Cells were cultured in MEM supplemented with 4% fetal bovine serum (HyClone, Logan, Utah), 2 mM L-glutamine, 100 U of penicillin G/ml, 100 μg of streptomycin sulfate/ml, and 20 μg of amphotericin B (Fungizone)/ml.

Quantitation of HCMV in semen samples by plaque assay. Quantitative culture of HCMV from semen samples was performed by the Virology Research Laboratory using a viral plaque assay (titration). Semen samples were initially diluted 1:10 in MEM and sonicated for 30 s for lysis. The sonicate was filtered through a 0.45-μm-pore-size filter, and appropriate dilutions were plated in duplicate in 24-well plates containing HFF. After incubation at 37°C for 3 h, the wells were washed and refed with MEM. Cells were cultured for 7 days, after which they were fixed and stained, plaques were counted, and the titer of HCMV in semen (expressed as PFU per milliliter) was determined.

Qualitative HCMV plaque assay in urine. Qualitative culture of HCMV (expressed as positive versus negative results) from urine samples was performed by the Virology Research Laboratory using a viral plaque assay similar to that described above for semen samples.

Quantitative HCMV DNA PCR assay. Quantitation of HCMV DNA was performed using an assay under development at that time by Roche Molecular Systems (Branchburg, N.J.). Reagents for PCR amplification were provided by Roche Molecular Systems, and the assays were performed according to the manufacturer's specifications. The limit of detection of the assay was 16 copies/ml, and the dynamic range was $>10^3$ copies/ml.

PCR amplification was performed in a 96-well plate by using an ABI Thermal Cycler 9600 (Perkin-Elmer Applied Biosystems, Foster City, Calif.), with the following amplification conditions: 50°C for 10 min; amplification at 96°C for 30 s and 65°C for 30 s for 30 cycles; and a hold at 72°C for no more than 2 h. Quantitative detection of the PCR products was based on colorimetric detection of an avidin-horseradish peroxidase complex bound to the biotin moiety of the HCMV DNA primer.

Determination of HCMV sensitivity to 1263W94 by plaque reduction assay. HCMV isolates from semen and urine samples were shipped either as growing, infected cells or as frozen stocks. Infected cells were plated on MRC-5 cells and grown for at least 2 passages until 70 to 90% of cells showed characteristic HCMV cytopathic effects. Infected cell stocks were made and titered.

Plaque reduction assays were performed to determine whether clinical isolates had acquired resistance to 1263W94. Clinical isolates were analyzed as cell-associated virus, and AD169 was analyzed as cell-free supernatant virus. Drug concentrations were tested in triplicate, and the mean values were determined for each clinical isolate at each concentration. 1263W94 was assayed at seven concentrations, ranging from 0.01 to 30 μM, as previously described (14).

RESULTS

Subject population. Seventy-eight subjects were enrolled in the study. Of these, 28 were assigned to the main group and 50 were assigned to the satellite group. The number of subjects included in each of the 1263W94 dose cohorts is shown in Table 1. The 62 subjects receiving 1263W94 and the 16 subjects receiving the placebo were similar in demographic characteristics, in $CD4^+$ cell count, and in Centers for Disease Control and Prevention (CDC) classification status at baseline, as shown in Table 1.

Of the 78 subjects enrolled in the study, 70 completed the 28-day dosing period of the study. Eight subjects withdrew from the study prematurely. Of these eight, six subjects (three in the 200-mg t.i.d. cohort and one each in the 600-, 900-, and 1,200-mg b.i.d. cohorts) discontinued due to adverse events, consisting of grade-2 rash (five subjects) and sinusitis (one subject). A seventh subject (in the 900-mg b.i.d. cohort) was withdrawn from the study after the day-1 hematology results indicated neutropenia (400 cells/mm^3), and an eighth subject (in the 900-mg b.i.d. cohort) withdrew from the study on day 2 due to anxiety about the serial PK sampling.

Concomitant medications. Subjects were stable on all chronically administered therapy for HIV and opportunistic infections for at least 1 month prior to receiving 1263W94. Most of the subjects were receiving concomitant medications that were CYP3A4 inhibitors, such as protease inhibitors and antifungal agents. One subject received efavirenz, a CYP3A4 inducer.

PK analysis. Descriptive statistics for plasma 1263W94 PK parameters are displayed in Table 2. There was a dose-proportional increase in plasma 1263W94 AUC_∞, C_{max}, and $AUC_{24,ss}$ over the dose range tested, as displayed in Fig. 1. The slope estimates (and 90% CI) for plasma 1263W94 parameters were as follows: AUC_∞, 1.12 (0.98, 1.27); $AUC_{24,ss}$, 0.95 (0.84, 1.06); and C_{max}, 0.98 (0.85, 1.10).

TABLE 1. Demographic and baseline characteristics

Characteristics[a]	Value for dosing group						
	100 mg t.i.d. (n = 11)	200 mg t.i.d. (n = 12)	400 mg t.i.d. (n = 10)	600 mg b.i.d. (n = 7)	900 mg b.i.d. (n = 12)	1,200 mg b.i.d. (n = 10)	Placebo (n = 16)
Age (yr)	37.0 (28.0, 57.0)	44.0 (32.0, 64.0)	40.0 (32.0, 51.0)	36.0 (30.0, 42.0)	41.5 (34.0, 53.0)	46.0 (28.0, 51.0)	40.0 (33.0, 49.0)
Race							
Caucasian	7 (64)	10 (83)	8 (80)	7 (100)	10 (83)	8 (80)	14 (88)
Black	2 (18)	1 (8)	1 (10)	0	1 (8)	0	1 (6)
Asian	0	0	0	0	1 (8)	0	0
Other	2 (18)	1 (8)	1 (10)	0	0	2 (20)	1 (6)
Ht (cm)	177.0 (170.0, 185.0)	177.5 (167.0, 195.0)	181.0 (170.0, 195.0)	173.0 (167.0, 188.0)	176.5 (144.0, 185.0)	171.5 (152.0, 195.0)	177.0 (168.0, 193.0)
Wt (kg)	79.1 (56.0, 105.5)	74.5 (60.9, 83.0)	83.0 (61.0, 121.5)	74.5 (57.9, 96.2)	78.9 (64.7, 101.0)	78.9 (68.2, 104.0)	81.0 (60.0, 98.2)
$CD4^+$ cell count (cells/mm^3)	110.0 (34.0, 256.0)	132.5 (9.0, 420.0)	130.5 (48.0, 308.0)	135.0 (7.0, 240.0)	98.0 (50.0, 189.0)	93.0 (50.0, 189.0)	93.5 (40.0, 210.0)
CDC classification							
Asymptomatic	0	1 (8)	1 (10)	0	0	0	2 (13)
Symptomatic	3 (27)	2 (17)	3 (30)	1 (14)	1 (8)	0	0
AIDS indicator	8 (73)	9 (75)	6 (60)	6 (86)	11 (92)	10 (100)	14 (88)

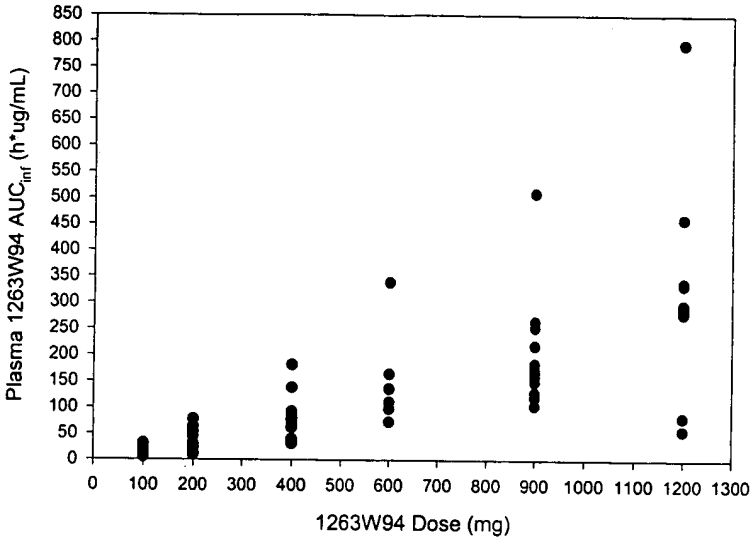
[a] Values for age, height, weight, and $CD4^+$ cell count are given as median (minimum, maximum). Values for race and CDC classification are given as the number (percentage) of subjects with the particular characteristic.

TABLE 2. Plasma 1263W94 PK parameters[a] at days 1 and 28

Dosing group	AUC_∞ (μg·h/ml) (day 1)	$AUC_{\tau,ss}$ (μg·h/ml) (day 28)	C_{max} (μg/ml) (day 1)	$C_{max,ss}$ (μg/ml) (day 28)	$C_{min,ss}$ (μg/ml) (day 28)	$C_{avg,ss}$ (μg/ml) (day 28)	T_{max}[b] (h) (day 1)	$T_{max,ss}$[b] (h) (day 28)	$t_{1/2}$ (h^{-1}) (day 1)
100 mg t.i.d.[c]	16.04 (10.56, 24.34)	15.58 (11.13, 21.80)	3.50 (2.57, 4.75)	3.98 (2.85, 5.56)	0.65 (0.35, 1.18)	1.95 (1.39, 2.73)	1.49 (0.50, 3.08)	2.00 (0.98, 3.02)	5.25 (3.99, 6.91)
200 mg t.i.d.[d]	29.75 (19.01, 46.58)	23.51 (12.76, 43.32)	7.10 (4.94, 10.20)	6.55 (4.26, 10.06)	0.79 (0.24, 2.53)	2.94 (1.59, 5.41)	1.75 (1.00, 3.00)	1.75 (1.00, 3.00)	4.52 (3.62, 5.62)
400 mg t.i.d.[e]	76.43 (52.65, 110.95)	74.47 (58.63, 94.60)	16.89 (12.78, 22.33)	18.45 (14.51, 23.45)	4.00 (2.97, 5.39)	9.31 (7.33, 11.82)	1.50 (1.00, 4.00)	1.50 (1.00, 2.00)	5.95 (4.35, 8.13)
600 mg b.i.d.[f]	134.45 (77.02, 234.71)	147.19 (82.76, 261.77)	25.40 (15.68, 41.14)	31.21 (18.40, 52.94)	4.12 (1.49, 11.37)	12.27 (6.90, 21.81)	2.00 (1.00, 3.00)	1.50 (1.00, 3.00)	6.83 (4.62, 10.08)
900 mg b.i.d.[g]	183.95 (140.11, 241.50)	209.69 (139.33, 315.57)	34.46 (29.41, 40.37)	41.01 (30.15, 55.76)	6.44 (3.06, 13.56)	17.47 (11.61, 26.30)	1.50 (1.00, 3.00)	1.50 (0.50, 3.00)	5.34 (4.36, 6.54)
1,200 mg b.i.d.[c]	228.27 (132.61, 392.96)	225.41 (155.36, 327.05)	30.83 (21.78, 43.64)	41.26 (31.06, 54.80)	4.04 (1.58, 10.33)	18.78 (12.94, 27.26)	2.50 (1.00, 3.00)	1.50 (1.00, 4.00)	6.40 (4.53, 9.06)

[a] Values are geometric means (95% CI), except for T_{max} and $T_{max,ss}$.
[b] Values are medians (with ranges in parentheses).
[c] n = 10 for day 1; n = 11 for day 28.
[d] n = 12 for day 1; n = 6 for day 28.
[e] n = 10 for day 1; n = 9 for day 28.
[f] n = 6 for day 1; n = 5 for day 28.
[g] n = 12 for day 1; n = 8 for day 28.

a



b

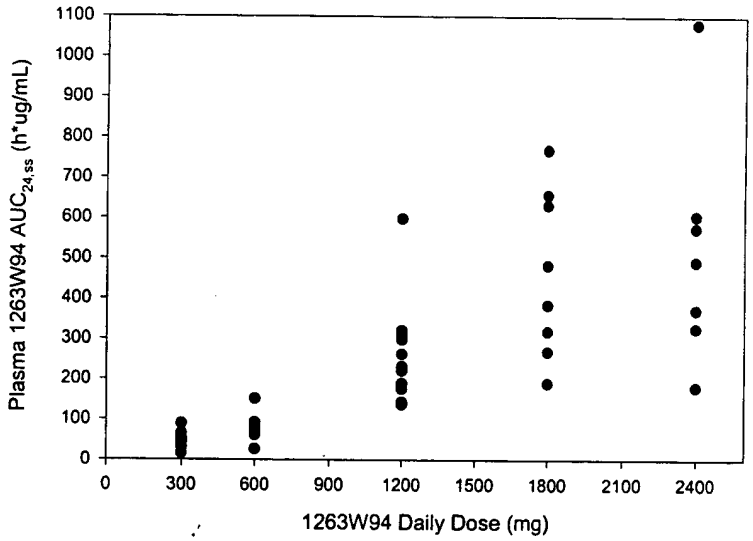


FIG. 1. Plots of dose versus plasma 1263W94 AUC_{∞} (a) and $AUC_{24,ss}$ (b).

1263W94 demonstrated linear PK, with steady-state plasma 1263W94 profiles predictable based on single-dose data as demonstrated by the ratio (and 90% CI) of $AUC_{\tau,ss}$ to AUC_{∞}. The ratio of plasma 1263W94 $AUC_{\tau,ss}$ to AUC_{∞} was close to 1, and the associated 90% CI was relatively balanced on either side of 1, for each of the doses administered.

The percentage of the dose that was excreted in urine as 1263W94 was minimal; on average, less than 3% of the parent compound was eliminated in urine. Semen 1263W94 concentrations increased with increasing doses. Median 1263W94 concentrations in semen (with the range and number of subjects given in parentheses) were as follows: for the 100-mg t.i.d. cohort, 1.67 μg/ml (0.30 to 3.53; n = 9); for the 200-mg t.i.d. cohort, 2.96 μg/ml (2.54 to 3.09; n = 4); for the 400-mg t.i.d. cohort, 8.41 μg/ml (3.62 to 25.57; n = 9); and for the 900-mg b.i.d. cohort, 11.85 μg/ml (3.42 to 21.77; n = 8). A semen 1263W94 concentration of 6.22 μg/ml was measured for a single subject in the 600-mg b.i.d. cohort.

Quantitation of HCMV in semen and whole blood. Figure 2 presents changes from baseline in semen HCMV amounts (based on plaque assays and PCR assays) for subjects in the main group at days 4 (when applicable), 7, 14, 21, and 28. Based on HCMV titers, subjects in the 200-mg t.i.d. (n = 5) and the 400-mg t.i.d. (n = 6) cohorts experienced the greatest decreases in HCMV amounts, with a mean decrease at day 28 of 3.7 $\log_{10}$ PFU/ml (standard deviations [SD], 0.96 for the

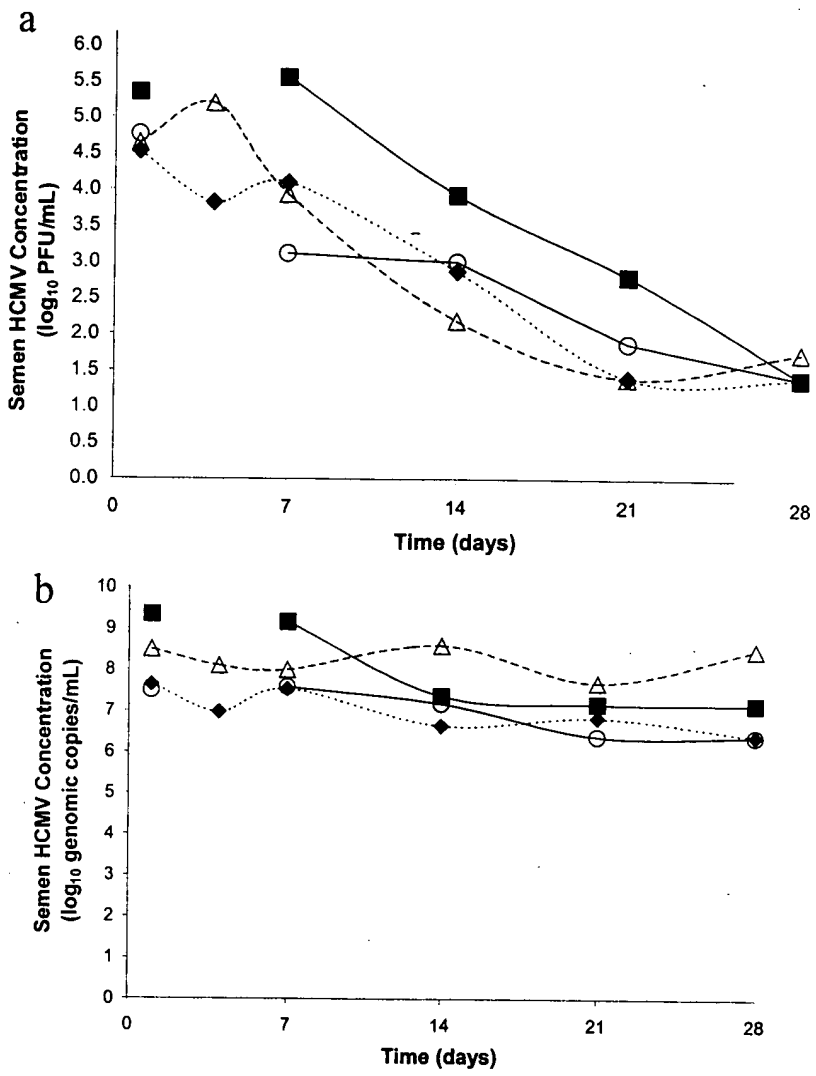


FIG. 2. (a) Median concentration-time profiles of HCMV in semen, measured in PFU per milliliter by using plaque assays. Symbols: ○, 100 mg t.i.d. (n = 7); ■, 200 mg t.i.d. (n = 5); ◆, 400 mg t.i.d. (n = 6); △, 600 mg b.i.d. (n = 6). (b) Median concentration-time profiles of HCMV DNA in semen, measured in log_{10} copies per milliliter by using PCR analysis. Symbols: ○, 100 mg t.i.d. (n = 7); ■, 200 mg t.i.d. (n = 4); ◆, 400 mg t.i.d. (n = 5); △, 600 mg b.i.d. (n = 2).

200-mg t.i.d. cohort and 1.08 for the 400-mg t.i.d. cohort), while subjects in the 100-mg t.i.d. cohort (n = 7) experienced a mean (±SD) decrease of 2.9 (±0.79) log_{10} PFU/ml and subjects in the 600-mg b.i.d. (n = 6) cohort experienced a mean (±SD) decrease of 3.3 (±1.32) log_{10} PFU/ml. Quantitative reductions in semen HCMV DNA amounts and the differences between the cohorts were less pronounced than for HCMV amounts as measured by titers. At day 28, mean decreases from baseline in HCMV DNA amounts were 1.1 log_{10} copies/ml for the 100-mg t.i.d. cohort (n = 7), 1.5 log_{10} copies/ml for the 200-mg t.i.d. cohort (n = 4), and 1.3 log_{10} copies/ml for both the 400-mg t.i.d. (n = 5) and 600-mg b.i.d. (n = 2) cohorts.

The numbers (and percentages) of subjects in each cohort with ≥2-log_{10}-unit reductions from baseline in semen HCMV titers at day 28 were as follows: 6 of 7 subjects (86%) in the 100-mg t.i.d. cohort, 5 of 5 subjects (100%) in the 200-mg t.i.d. cohort, 6 of 6 subjects (100%) in the 400-mg t.i.d. cohort, and 5 of 6 subjects (83%) in the 600-mg b.i.d. cohort. Only a single subject in each cohort had a ≥2-log_{10}-unit reduction in semen HCMV DNA concentrations from baseline to day 28.

Only one subject, who received 1263W94 at 600 mg b.i.d., had quantifiable HCMV DNA in whole blood at baseline (4.81 log_{10} copies/ml). In this subject HCMV DNA amounts decreased to below the limit of detection by the PCR assay (<1.2 log_{10} copies/ml) by day 28.

Detection of HCMV in the urine. The numbers (and percentages) of subjects in each cohort whose qualitative HCMV cultures showed positive results at baseline and negative results in the last two on-treatment measurements (days 21 and 28) were as follows: 2 of 7 (29%) in the 100-mg t.i.d. cohort, 1 of 5 (20%) in the 200-mg t.i.d. cohort, 3 of 6 (50%) in the

400-mg t.i.d. cohort, and 1 of 6 (17%) in the 600-mg b.i.d. cohort.

HCMV sensitivity to 1263W94 by plaque reduction assay. Ninety-two HCMV isolates were obtained from day-1, day-28, or day-56 cultures of semen and urine samples. One subject provided a day-11 semen sample, which was positive for HCMV and was included in the analysis. The 92 isolates analyzed were from 31 subjects in the 100-mg t.i.d., 200-mg t.i.d., and 400-mg t.i.d. cohorts and included 58 semen samples (24 from day 1, 1 from day 11, 15 from day 28, and 18 from day 56) and 34 urine samples (13 from day 1, 9 from day 28, and 12 from day 56).

The median 50% inhibitory concentration (IC_{50}) of 1263W94 for the clinical isolates was 0.27 μM (range, 0.05 to 0.88 μM). There were no significant differences in the median IC_{50} between the day-1 samples and the day-28 or day-56 samples. The median IC_{50} for wild-type strain AD169, determined from 10 separate plaque reduction assays, was 0.55 μM (range, 0.23 to 0.78 μM).

Because the clinical isolates tested might contain mixed populations of drug-susceptible virus and treatment-emergent drug-resistant virus selected by exposure to 1263W94, additional assays were performed to determine the level of resistant virus in a mixed viral population necessary to produce an increase in the IC_{50}. Mixtures of wild-type AD169 and the 1263W94-resistant strain 2916^r were assayed for 1263W94 susceptibility by using plaque reduction assays. IC_{50}s did not increase with a mixture containing 10% resistant virus but increased 3.3- and 16-fold with mixtures containing 25 and 50% resistant virus, respectively. These results suggested that emergence of 1263W94-resistant variants comprising as little as 25% of the viral population would have been detected in plaque reduction assays of the clinical isolates.

Safety and tolerability. The majority of subjects enrolled in the study, including 60 of 62 subjects (97%) who received 1263W94 and 10 of 16 subjects (63%) who received the placebo, experienced at least one adverse event during the study period. Most of the subjects reported adverse events that were neurological, such as taste disturbances and headache, or gastrointestinal, such as diarrhea and nausea. Adverse events reported by ≥10% of subjects in either the 1263W94 or the placebo group, and the numbers (and percentages) of subjects reporting the event in the 1263W94 and placebo groups, respectively, were as follows: taste disturbances, 51 (82%) and 3 (19%); headache, 13 (21%) and 3 (19%); diarrhea, 16 (26%) and 2 (13%); nausea, 14 (23%) and 2 (13%); rash, 12 (19%) and 1 (6%); pruritus, 12 (19%) and 1 (6%); fever, 7 (11%) and 0; exacerbation of fatigue, 6 (10%) and 2 (13%); vomiting, 5 (8%) and 2 (13%); constipation, 0 and 2 (13%); and upper respiratory tract infection, 2 (3%) and 2 (13%). Diarrhea and taste disturbances appeared to be dose related. After approximately 7 to 12 days of therapy, five subjects receiving 1263W94 prematurely discontinued the study due to a drug-related diffuse maculopapular rash of moderate (grade-2) intensity. Two of these subjects received 200 mg t.i.d., and one each received 600, 900, and 1,200 mg b.i.d. Four of these subjects had a history of allergic reaction to other drugs. After discontinuation of the study drug, the rash resolved within approximately 1 to 4 days without sequelae.

Two serious non-drug-related adverse events were reported during the study. The first event was cholecystitis, for which the subject underwent a cholecystectomy. The second event was a single episode of pulmonary thromboembolism. The symptoms of pulmonary thromboembolism (dyspnea and chest pain) resolved within 2 weeks of hospital admission and initiation of appropriate therapy. Both events occurred in subjects who received the placebo.

Clinical laboratory values for subjects who received 1263W94 and subjects who received the placebo were not significantly different, and there were no dose-related trends in clinical laboratory values. Decreases in lymphocytes and increases in total protein were consistent with the HIV-infected status of the subjects, and modest decreases in hemoglobin were consistent with the phlebotomy requirements of the protocol. Hemoglobin values increased to screening values by the 4-week poststudy visit.

DISCUSSION

There is a need to develop effective and safe oral therapies for the treatment and prevention of HCMV disease. 1263W94 is a benzimidazole riboside with potent and selective inhibition of HCMV in vitro and with limited cytotoxic and toxicologic effects based on in vitro and in vivo screenings (1). Pre-clinical studies have indicated that 1263W94 has good oral bioavailability (9).

This study was designed to evaluate the PK, anti-HCMV activity, safety, and tolerability of 1263W94 administered as multiple oral doses to HIV-infected subjects with asymptomatic HCMV shedding in urine and semen over a 28-day treatment period. Anti-HCMV activity was evaluated by measuring HCMV titers in semen (5, 10) and by measuring HCMV DNA amounts in semen and whole blood by PCR. Urine cultures were not used for quantitation of HCMV, because the urinary tract is a closed system, and variations in urine volume may affect accurate quantitation of HCMV.

Semen HCMV titration has proven useful in demonstrating in vivo anti-HCMV activity and in selecting clinically useful doses of HCMV therapies. For example, cidofovir was more effective at reducing semen HCMV titers at a weekly dose of 5.0 mg/kg of body weight than at a weekly dose of 3.0 mg/kg (10), and the higher dose was also more effective in clinical trials (11, 12)

Over 28 days of dosing, 1263W94 demonstrated in vivo anti-HCMV activity in semen at all of the dosage regimens tested (100, 200, and 400 mg t.i.d., and 600 mg b.i.d.), with mean reductions in semen HCMV titers of 2.9 to 3.7 $\log_{10}$ PFU/ml among these four regimens. The lowest dosage regimen appeared to have less anti-HCMV activity than the three higher dosage regimens; however, the reductions in HCMV titers for all of the 1263W94 dosage regimens tested compare favorably with results reported for the approved doses of cidofovir (5 mg/kg) (10).

A greater change from baseline in HCMV load following administration of 1263W94 was measurable by culture using plaque titration than by quantitative PCR. An explanation could be that synthesis of DNA continues to some degree in the presence of 1263W94 but that intact viable virus is not produced. Thus, the antiviral effect of 1263W94 could initially

be more evident by culture of viable virus than by quantitation of HCMV DNA synthesis. Also, the quantitative reduction in viral DNA may lag behind plaque reduction. If we had continued to assay viral DNA in semen at weeks 5 and 6 instead of stopping at 28 days, we might have seen greater log reductions in HCMV DNA concentrations.

Although relatively high concentrations of 1263W94 were detected in semen, the anti-HCMV effect in semen as measured by plaque assay is not attributable to drug carryover into viral cultures, as shown by the following observations. (i) Semen HCMV titers decreased progressively from day 1 to day 28 (see Fig. 2); a carryover effect should have been equally apparent in all sequential samples. (ii) HCMV DNA levels in semen also decreased over the 28-day treatment period. The PCR assay is a direct measure of viral DNA at the time of specimen collection and is not subject to inhibition by 1263W94 during the assay.

There was a dose-proportional increase in plasma 1263W94 AUC_{∞}, C_{max}, and $AUC_{24,ss}$ over the dose range tested. 1263W94 demonstrated linear PK, with steady-state plasma profiles predictable based on single-dose data. Several of the concomitant medications that subjects received for treatment of HIV and opportunistic infections were inhibitors, inducers, and/or substrates of CYP3A4, the isoenzyme primarily responsible for 1263W94 metabolism. The effects of these drugs on 1263W94 PK were not assessed.

1263W94 was generally safe and reasonably well tolerated during the 28 days of dosing. Six subjects prematurely discontinued the study drug due to adverse events: five cases of rash and one of sinusitis. Two subjects, both receiving the placebo, reported serious non-drug-related adverse events during the study. Taste disturbance was the most frequently reported adverse event. Taste disturbance, diarrhea, nausea, rash, pruritus, and fever were reported by a higher percentage of subjects receiving 1263W94 versus the placebo, and taste disturbance and diarrhea appeared to be dose related. Overall, 1263W94 showed a favorable profile with regard to safety, tolerability, PK, and anti-HCMV effect.

The absence of resistance in isolates obtained at day 28 of treatment (or 28 days later) is encouraging but is similar to previous data for ganciclovir and cidofovir (2, 4). In order to evaluate the risk of developing resistance, it would be necessary to assess IC_{50}s for isolates from patients receiving 1263W94 for ≥90 days (4, 8).

The oral bioavailability of 1263W94, the duration and magnitude of its antiviral effect, and the lack of dose-limiting toxicity make it very attractive as a potential anti-HCMV therapeutic and prophylactic agent. The reductions in HCMV titers for all of the 1263W94 dosage regimens tested compare favorably with results reported for the approved anti-HCMV agents. In addition, the relatively benign toxicity profile of 1263W94 and the specific absence of nephrotoxicity or myelotoxicity suggest a potential role for this agent for patients receiving solid-organ and bone marrow transplants as well as for patients with HIV.

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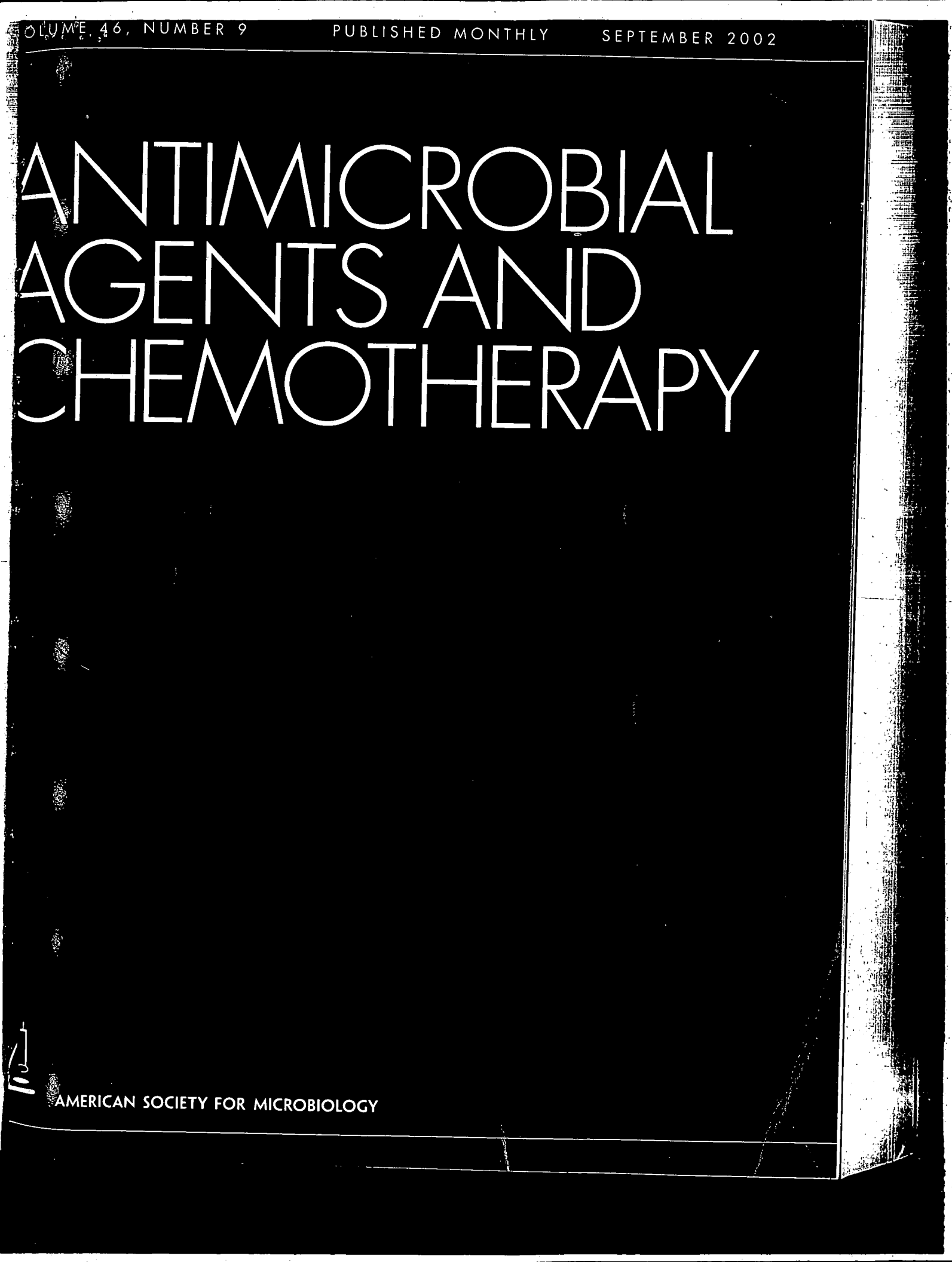

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ARTICLES

Feasibility of a High-Flux Anticancer Drug Screen Using a Diverse Panel of Cultured Human Tumor Cell Lines

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We describe here the development and implementation of a pilot-scale, in vitro, anticancer drug screen utilizing a panel of 60 human tumor cell lines organized into subpanels representing leukemia, melanoma, and cancers of the lung, colon, kidney, ovary, and central nervous system. The ultimate goal of this disease-oriented screen is to facilitate the discovery of new compounds with potential cell line-specific and/or subpanel-specific antitumor activity. In the current screening protocol, each cell line is inoculated onto microtiter plates, then preincubated for 24-28 hours. Subsequently, test agents are added in five 10-fold dilutions and the culture is incubated for an additional 48 hours. For each test agent, a dose-response profile is generated. End-point determinations of the cell viability or cell growth are performed by in situ fixation of cells, followed by staining with a protein-binding dye, sulforhodamine B (SRB). The SRB binds to the basic amino acids of cellular macromolecules; the solubilized stain is measured spectrophotometrically to determine relative cell growth or viability in treated and untreated cells. Following the pilot screening studies, a screening rate of 400 compounds per week has been consistently achieved. [J Natl Cancer Inst 83:757–766, 1991]

The National Cancer Institute is implementing a new investigational, in vitro, disease-oriented, drug-discovery screen. Other publications describe the overall concept and rationale for this screen (*1-3*) and various aspects of its technical evolution (*4-7*). The purpose of the screen is to provide for the initial evaluation of more than 10 000 new substances per year for cytotoxic and/or growth-inhibitory activity against a wide diversity of tumor types and to allow the detection of possible tumor-type-specific sensitivity. The data from such an in vitro primary screen will allow selection of the most sensitive cell line(s) for subsequent in vivo xenograft testing of compounds of interest. While the concept is simple, the technical challenges for implementation of such a screen are greatly magnified by the required scope of the program.

The goal of the program is to implement the full-scale screen with a capacity for testing new substances at a rate of more than 10 000 per year against a broadly representative panel of 100 or more human tumor cell lines. In moving toward this goal, we initiated the pilot-scale screen outlined here, incorporating experience gained from previous laboratory-scale research and development efforts. With the pilot screen we have explored a variety of conceptual issues, as well as technical and managerial issues critically involved in a scale-up to the full screen. This paper describes our experiences with the pilot screen and its evolution through a variety of technical stages, including comparative evaluations of two types of tetrazolium assays (*4,5*) and an assay using the protein-binding dye sulforhodamine B (SRB) (*6*), all of which are microculture assays to measure cell viability and cell growth.

The results from operation of the pilot screen have been evaluated both continuously and retrospectively with a particular view toward determining the feasibility of implementation of the full-scale screen.

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Materials and Methods

Cell Lines

The human tumor cell lines currently used in the pilot screen have been presented previously (*7*); details of their origin and characterization are described elsewhere (Stinson SF, Alley MC, Kopp WJ, et al: manuscript submitted). Parallel to the implementation and operation of the pilot screen, a major effort continues for the acquisition and evaluation of new cell lines for possible addition or substitution into the panel. Ultimately, the cell line panel may contain as many as twice the current number of lines, with each disease-related subpanel containing 10-15 representative lines (*3*). However, for purposes of the pilot screen, we have focused on a fixed set of 60 lines (Table 1).

Criteria for selection of a cell line for use in the interim panel were as follows:

(*a*) adaptability to growth on a single medium (RPMI-1640 plus 5% fetal bovine serum and 2 m*M* glutamine);
(*b*) a negative test for mycoplasma and mouse antibody production;
(*c*) isoenzyme and karyotype profiles verifying the human origin of the cells;
(*d*) mass doubling time that allows for harvesting of approximately 3×10^7 cells twice a week; and
(*e*) suitability for use with microculture assays.

Once a line had been established as suitable, the number of cells was massively expanded in a minimal number of passages, and the cells were cryopreserved in a large repository of ampules, each containing 1×10^6 cells, to provide a consistent, long-term frozen stock for future use (*4*). Within the primary drug evaluation laboratory, new cell line stock samples are thawed as each cell line culture used for screening approaches its 20th passage or if there is a noticeable change in growth, morphology, or drug-response characteristics. Once the growth of the new stock is established at the second or third passage, the older passage line is replaced with the new stock for use in the screening laboratory.

Cell Line Maintenance

Major considerations with respect to cell line maintenance include (*a*) the necessity of continually producing approximately 3×10^7 cells for biweekly inoculation for the pilot screen, (*b*) the potential for cross-contamination of cell lines, and (*c*) the possibility of low-grade microbial contamination. Individual technicians are therefore assigned only six specific cell lines, which they monitor continually for growth characteristics in tissue-culture flasks and microculture plates, for behavior in the microculture assays, and for microscopic appearance. Furthermore, the use of a limited number of passages from a frozen-stock vial also helps prevent long-term cross-contamination of a cell line. Cells are grown and passaged in antibiotic-free growth medium to ensure freedom from microbial contaminants.

Cells are maintained in multiple T150 tissue-culture flasks. Cells for each inoculation day are maintained separately (no common reagents) and passaged on separate days to prevent catastrophic loss of growing cell line stocks to microbial contamination. Additional backup flasks of cells are also maintained. For each cell line, the seeding density per flask is determined for production of a healthy culture of 70%-90% confluency after 7 days for continued routine maintenance or after 4 or 5 days for the microculture assay. These seeding densities are then utilized twice a week to maintain sufficient cells for routine culture and for anticancer drug screening.

Preparation and Inoculation of Cells

All of the adherent cell lines are detached from the culture flasks by addition of 2-3 mL of 0.05% trypsin-EDTA (GIBCO Laboratories, Grand Island, NY). Thereafter, trypsin is inactivated by addition of 10 mL of 5% serum-containing RPMI-1640 medium. Cells are separated into single-cell suspensions by a gentle pipetting action, then counted using trypan-blue exclusion on a hemacytometer or by a Coulter counter, which is used when viability as determined by trypan-blue exclusion is routinely greater than 97% and the cells maintain suspension as single cells. After counting, dilutions are made to give the appropriate cell densities for inoculation onto the microtiter plates. Cells are inoculated in a volume of 100 µL per well at densities between 5000 and 40 000 cells per well (Table 1); the basis for selection of the particular inoculation densities for each cell line is described in the Results section. A 100-µL aliquot of complete medium is added to cell-free wells. Cells from all cell lines are counted, diluted, and inoculated onto microculture plates within a 4-hour period on 2 days each week. The microtiter plates containing the cells are preincubated for approximately 24 hours at 37°C to allow stabilization prior to addition of drug.

Solubilization and Dilution of Samples

For initial screening of pure compounds, each agent is routinely tested at five 10-fold dilutions, starting from a maximum concentration of 10^{-4} *M*. Alternatively, a maximum of 10^{-3} *M* may be selected if solubility permits, if the higher upper limit is desired, and if sufficient compound is available. Alternative initial concentrations may also be utilized for retesting, particularly for potent cytotoxins, for compounds of limited solubility, or for nonroutine detailed comparisons of selected compounds when previous information is available. Crude extracts of natural products are tested at five threefold dilutions, starting at an upper limit of 250 µg/mL regardless of solubility, ie, particulate matter may be present. All samples are initially solubilized in dimethyl sulfoxide (DMSO) or water at 400 times the desired final maximum test concentration. Drug stocks are not filtered or sterilized, but microbial contamination is controlled by addition of gentamicin to the drug diluent. Multiple aliquots are stored frozen at –70°C to provide uniform samples for initial tests as well as for retests, if required. Just prior to preparation of the drug dilutions in cell-culture medium, these frozen concentrates are thawed at room temperature for 5 minutes. The concentrates are then diluted with complete medium containing 50 µg/mL gentamicin to twice the desired final concentrations.

Drug Incubation

Immediately after preparation of these intermediate dilutions, 100-µL aliquots of each dilution are added to the appropriate microtiter plate wells according to the format in Fig 1, in which

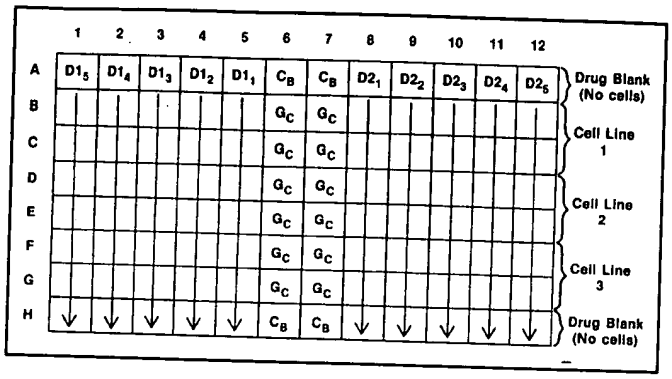

Fig 1. Microtiter plate format used for screening shows three cell lines and two test agents (D1 and D2), each inoculated at five concentrations (D1$_1$-D1$_5$ and D2$_1$-D2$_5$). One plate holds six dose-response experiments with quadruplicate growth control (G_C) wells, four control background (C_B) wells, and duplicate test wells, for each dose-response set. Letters A-H and Nos. 1-12 represent the microtiter plate map.

three cell lines are inoculated per plate. As the microtiter wells already contain the cells in 100 μL of medium, the final drug concentration tested is 50% of that in the intermediate dilutions. Agents are then added immediately to the cultures in the microtiter plates. During development of these procedures, drug incubation times were 1, 2, 3, 4, or 6 days at 37°C in an atmosphere of 5% CO_2 and 100% relative humidity. The plates were then assayed for cellular growth and viability by microculture assay—either by one of the two types of tetrazolium assay or by the SRB assay. In the current screening procedure, the cultures are incubated with test agents for 2 days, and the end point is measured by the SRB assay.

Microculture Tetrazolium Assay

The MTT assay is based on metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Details of this assay have been published previously (*4*), but certain modifications were made for use in the pilot screen. A 50-μL aliquot of MTT solution (1 mg/mL) in RPMI-1640 medium, with no serum or glutamine, is added directly to all the appropriate microtiter plate wells containing cells, complete growth medium, and test agents. The culture is then incubated for 4 hours to allow for MTT metabolism to formazan. After this time, the supernatant is aspirated and 150 μL of DMSO is added to dissolve the formazan. Plates are agitated on a plate shaker to ensure a homogeneous solution, and the optical densities are read on an automated spectrophotometric plate reader (model 312; Biotech Research Laboratories, Inc, Rockville, Md) at a single wavelength of 550 nm.

The XTT assay is based on the metabolic reduction of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT). The detailed methodology is presented elsewhere (*5*). Briefly, 1 mg/mL XTT is mixed with phenazine methosulfate (7.65 μg/mL) immediately prior to its addition to microtiter plates. Fifty microliters of the mixture is added to each well and incubated for 2-4 hours, depending on the metabolizing characteristics of the individual cell lines. Each plate is agitated for 2-3 minutes on a plate shaker; then optical densities are read on an spectrophotometric plate reader at a single wavelength of 450 nm.

Sulforhodamine B Assay

The detailed methodology for the SRB assay is presented elsewhere (*6*). Briefly, adherent cell cultures are fixed in situ by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) (final concentration, 10% TCA) and incubating for 60 minutes at 4°C. The supernatant is then discarded, and the plates are washed five times with deionized water and dried. One hundred microliters of SRB solution (0.4% wt/vol in 1% acetic acid) is added to each microtiter well, and the culture is incubated for 10 minutes at room temperature. Unbound SRB is removed by washing five times with 1% acetic acid. Then the plates are air-dried. Bound stain is solubilized with Tris buffer, and the optical densities are read on an automated spectrophotometric plate reader at a single wavelength of 515 nm.

For suspensions of cell cultures (eg, leukemias), the method is the same, except that at the end of the drug-incubation period, the settled cells are fixed to the bottom of the microtiter well by gently adding 50 μL of 80% cold TCA (final concentration, 16% TCA).

Data Calculations

Unprocessed optical density data from each microtiter plate are automatically transferred from the plate reader to a microcomputer, where the background optical density (OD) measurements (ie, complete medium plus stain, minus cells) are subtracted from the appropriate control well values and where the appropriate drug-blank measurements (ie, complete medium plus test compound dilution plus stain, minus cells) are subtracted from the appropriate test well values. The values for mean ± SD of data from replicate wells are calculated. Data are expressed in terms of %T/C [(OD of treated cells/OD of control cells) × 100], as a measure of cell viability and survival in the presence of test materials. Calculations are also made for the concentration of test agents giving a T/C value of 50%, or 50% growth inhibition (IC_{50}), and a T/C value of 10%, or 90% growth inhibition (IC_{90}).

With the SRB assay, a measure is also made of the cell population density at time 0 (the time at which drugs are added) from two extra reference plates of inoculated cells fixed with TCA just prior to drug addition to the test plates. Thus, we have three measurements: control optical density (C), test optical density (T), and optical density at time zero (T_0).

Using these measurements, cellular responses can be calculated for growth stimulation, for no drug effect, and for growth inhibition. If T is greater than or equal to T_0, the calculation is $100 \times [(T - T_0)/(C - T_0)]$. If T is less than T_0, cell killing has occurred and can be calculated from $100 \times [(T - T_0)/T_0]$ (*8*). Thus, for each drug-cell line combination, a dose-response curve is generated and three levels of effect are calculated. Growth inhibition of 50% (GI_{50}) is calculated from $100 \times [(T - T_0)/(C - T_0)] = 50$, which is the drug concentration causing a 50% reduction in the net protein increase in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from $T = T_0$, where the amount of protein at the end of drug incubation is equal to the amount at the beginning. The final calculation, LC_{50}, is the concentration of drug causing a 50% reduction in the measured protein at the end of the drug incubation, compared with that at the beginning,

indicating a net loss of cells following drug treatment. LC_{50} is calculated from $100 \times [(T - T_0)/T_0] = -50$.

Values are calculated for each of these parameters if the level of effect is reached, but if the effect is not reached or is exceeded, then the value is expressed as greater or less than the maximum or minimum drug concentration tested.

Computer Support and Automation

The projected goal of the National Cancer Institute (NCI) in vitro drug screen is to support the initial yearly evaluation of 10 000 or more new agents against as many as 100 or more cell lines. This would result in the generation of more than 1 million dose-response curves per year. Extensive computer support has necessarily been developed for each step of the screening process, from the shipping of an agent to the drug-preparation laboratory to the analysis and return of screening data to a supplier.

Agents to be screened are received by the drug-preparation laboratory, and the NCI drug identification code is entered into the computerized data management and monitoring system. After solubilization of the agents, details of the solubility and priority for testing are entered to update the database. In addition, bar-coded labels containing all the appropriate information are generated and attached to the storage vials containing the concentrates, which are then placed in interim storage at –70°C.

Screening-laboratory staff determine the available test capacity for the following week and enter this information into the mainframe database. Then, automated assignment of test agents to fill the scheduled requirements is performed according to preassigned priority status. Alternatively, assignments or reassignments may be performed manually.

Immediately prior to the day of an assignment, information is loaded via modem to microcomputers (Compaq 386-20 running an SCO XENIX operating system) in the screening laboratories. Labels are generated and printed by computer for each microculture plate. Each label contains an identification number that is specific to a particular cell line-drug-plate format.

After each technical task (ie, cell inoculation, addition of drug, or staining), the technicians confirm successful completion, using computer terminals available in each tissue-culture laboratory. This procedure allows for cross-checking of each step of the assay to ensure that the actual and assigned procedures are identical. Once an identity number has been entered, the computer system tracks it through all the technical steps so that when the optical densities are read spectrophotometrically, dose-response data can be calculated automatically and printed within minutes of the plate reading. At this point, there is provision for the manual elimination of suspicious data points by insertion of "reason codes" (eg, for microbial contamination, improper cell inoculation, or improper drug inoculation). These procedures are designed to prevent entry into the mainframe database of dose-response data known to be flawed for specific reasons.

In addition to these provisions for manual deletions to ensure quality control, a series of automated quality control parameters are performed by computer. These include "flags" for (*a*) variation exceeding 20%, as determined from the coefficient of variation (CV) of the mean of control optical densities ($n = 4$); (*b*) variation exceeding 25%, as determined from the CV of the mean of duplicate dose-response optical densities; (*c*) mean background optical densities greater than 10% of control; (*d*) mean control optical densities exceeding 3 SD of the mean optical densities for the six previous experiments for that cell line; and (*e*) a dose-response curve transecting the point of 50% growth inhibition more than once (reversal).

These codes constitute a "failure" of part or all of a dose-response determination and eliminate those data from further analysis. On a daily basis, data are copied to an on-site microcomputer for backup and laboratory analysis, then downloaded nightly to a large central computer (VAX 8820) for future detailed analysis, comparisons, and generation of screening-data reports for suppliers of the samples tested.

Procedure for Submitting New Cell Lines

Investigators who wish to submit cell lines for consideration of inclusion in the panel should contact:

Mr. Richard Camalier, Biologist
BTB, DTP, DCT, NCI-FCRDC
Building 321, Room 7
Fort Detrick, Frederick, MD 21702
Telephone (301) 846-5065
FAX (301) 846-5439

Results

Choice of Assay for Cell Growth and Viability

For the initial feasibility studies, the microculture tetrazolium assays (MTT and XTT) were utilized to determine cell growth and viability. During the evolution of the large-scale screen, however, certain technical limitations of these assays became especially apparent. As a result, we investigated alternative approaches, using protein and biomass stains. We found an assay using sulforhodamine B (SRB), a dye that binds to basic amino acids of cellular macromolecules, to be promising for purposes of this screen (*6*).

The major technical disadvantage of the microculture tetrazolium assays (MTT and XTT) is that the "moving-target" nature of the optical density, which is determined by formazan production, is a function of time, in addition to cell number. With the requirements to screen at least 10 000 agents per year and to compare each cell line against an agent in a single test, 1000 or more microculture plates must be evaluated for cell growth inhibition and viability on the same day. This presents a severe logistical challenge, especially at the stage of plate reading, as there is a very narrow window of time within which the tetrazolium plates must be processed.

The MTT microculture assay is a reasonably simple, sensitive, reproducible assay that exhibits good signal-to-noise ratios (*4*). However, the MTT procedure requires aqueous aspiration and DMSO-solubilization steps, making the procedure more time-consuming and potentially error-prone. Moreover, the use of large quantities of DMSO is undesirable in a busy screening-laboratory environment.

The XTT microculture assay is technically very simple and can be applied directly to suspension or monolayer cultures.

However, the XTT assay yields relatively poor signal-to-noise ratios and requires addition to the assay medium of an electron-coupling agent (phenazine methosulfate) representing yet another potential source of experimental variation. Furthermore, with the XTT assay, there was occasional formation of a crystalline material in the culture wells, which strongly interfered with the assay. This phenomenon was subsequently related in part to CO_2 variations and buffering instability (*9*) and was one of the factors leading to development of a new CO_2-independent culture medium (*10*) to obviate the problem.

The SRB assay has a stable end point, since the drug-exposed cells are fixed with TCA at the end of the incubation period (*6*). Once the cells are fixed and stained, there is no critical time restriction for subsequent determination of the SRB optical densities, which change less than 2% over a 7-day period if evaporation is restricted (data not shown). Furthermore, the SRB assay provides excellent signal-to-noise ratios. The technical manipulations required for staining are greater than for the tetrazolium assays, requiring up to 10 washing steps and two drying steps. However, these steps can be automated and, under the current protocol, can be readily scheduled around the activities of cell inoculation, addition of drug, and in situ staining of cells.

Comparisons of data from several hundred agents screened in parallel by MTT and SRB assays indicated that, under the experimental conditions employed and within the limits of the data analyses applied, the MTT and SRB assays generally yielded quite similar results (*11*). Therefore, the SRB procedure was subsequently selected for routine use in the pilot screen, principally because of the technical advantages described above.

Assay Parameters

Assay parameters refer here to the particular selections of (*a*) initial cell densities for inoculation and (*b*) drug incubation times. To avoid biasing the screen excessively toward detection of antiproliferative effects that might otherwise obscure interesting patterns of differential cytotoxicity, we have focused our developmental efforts on screening protocols incorporating relatively high initial inoculation densities and relatively short incubation times. Table 1 shows the specific cell inoculation densities used for each cell line; optimal initial cell densities were determined for each line as described in the following section.

As the cell line panel was expanded to include new lines, it became apparent that many cell lines were subject to severe nutrient depletion by the end of a 6-day incubation period. This was indicated by extreme acidity of the growth medium and peeling of the cells from the microculture plate well surface. An additional consideration was the effect of incubation time on cell doubling times. An investigation into the growth rate of cells inoculated into microculture plates was undertaken. Six cell lines with different growth rates were selected and their growth in the described protocol was examined. During days 1 and 2, the doubling time increased as expected, but between days 3 and 4, two of the six cell lines showed negative growth rates, indicating that the populations were dying faster than they were growing (data not shown).

Table 1. Human tumor cell lines and inoculation densities used in the NCI disease-oriented in vitro drug screen

Cell line	Cells/well	Cell line	Cells/well	Cell line	Cells/well
Lung cancer		***Colon cancer***		***CNS cancer***	
NCI-H23	20 000	HT29	5000	SNB-19	15 000
NCI-H226	20 000	HCC-2998	10 000	SNB-75	20 000
NCI-H322M	20 000	HCT-116	5000	SNB-78	20 000
NCI-H460	5000	SW-620	10 000	U251	7500
NCI-H522	15 000	COLO 205	15 000	SF-268	15 000
A549/ATCC	10 000	DLD-1	5000	SF-295	10 000
EKVX	20 000	HCT-15	10 000	SF-539	15 000
HOP-18	20 000	KM12	15 000	XF 498	20 000
HOP-62	15 000	KM20L2	10 000		
HOP-92	20 000			***Ovarian cancer***	
LXFL 529	10 000	***Melanoma***		OVCAR-3	10 000
DMS 114	20 000	LOX IMVI	5000	OVCAR-4	10 000
DMS 273	5000	MALME-3M	20 000	OVCAR-5	20 000
		SK-MEL-2	20 000	OVCAR-8	10 000
Renal cancer		SK-MEL-5	10 000	IGR-OV-1	10 000
UO-31	20 000	SK-MEL-28	10 000	SK-OV-3	20 000
SN12C	15 000	M19-MEL	10 000		
A498	20 000	UACC-62	10 000	***Leukemia***	
CAKI-1	10 000	UACC-257	20 000	CCRF-CEM	40 000
RXF 393	20 000	M14	15 000	K-562	5000
RXF 631	10 000			MOLT-4	30 000
ACHN	15 000			HL-60	20 000
786-0	10 000			RPMI-8226	20 000
TK-10	15 000			SR	30 000

To examine the difference between the results at 1, 2, 3, and 4 days of incubation, the SRB assay was used to perform a dose-response analysis on 20 standard drugs tested in six cell lines (A2780, HT29, HOP-62, OVCAR-5, SN12KI, and SK-MEL-5). A2780 and SN12KI have since been deleted from the cell line panel. The 20 agents were doxorubicin, amsacrine (AMSA), bleomycin, cisplatin, dibromodideoxymannitol (mitobronitol), gossypol, melphalan, mitomycin-C, retinoic acid, vinblastine, actinomycin (dactinomycin), carmustine (BCNU), chromomycin, cordycepin (3′-deoxyadenosine), fluorouracil (5-FU), homoharringtonine, methotrexate, phyllanthoside, tamoxifen, and etoposide (VP-16).

With the assays using 1-4 days of incubation, it is possible to determine a signal (optical density) at the time drug is added (T_0). Using these values, calculations can be made for the concentration of drug causing 15% growth inhibition (GI_{15}), 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell kill (LC_{50}). Fig 2 shows representative dose-response curves comparing 1, 2, 3, or 4 days' exposure of six cell lines to doxorubicin and BCNU. Table 2 shows a summary of the cellular response parameters calculated as a function of drug incubation time from such curves obtained for all 20 of the drugs used as standards. These data indicated that growth inhibition (GI_{15} and GI_{50}) by the selected standard clinical agents could be reliably determined at 2-4 days of drug exposure but that this determination was much less reliable after only 1 day of drug exposure. The more demanding end points of TGI and LC_{50} were less commonly achieved than the end points of GI_{15} and GI_{50}, but the 1- and 2-day incubations discriminated these parameters less well than the longer incubation periods. The TGI parameter was achieved in five or more cell lines in the 1- or 2-day assay only with doxorubicin, AMSA, BCNU, gossypol,

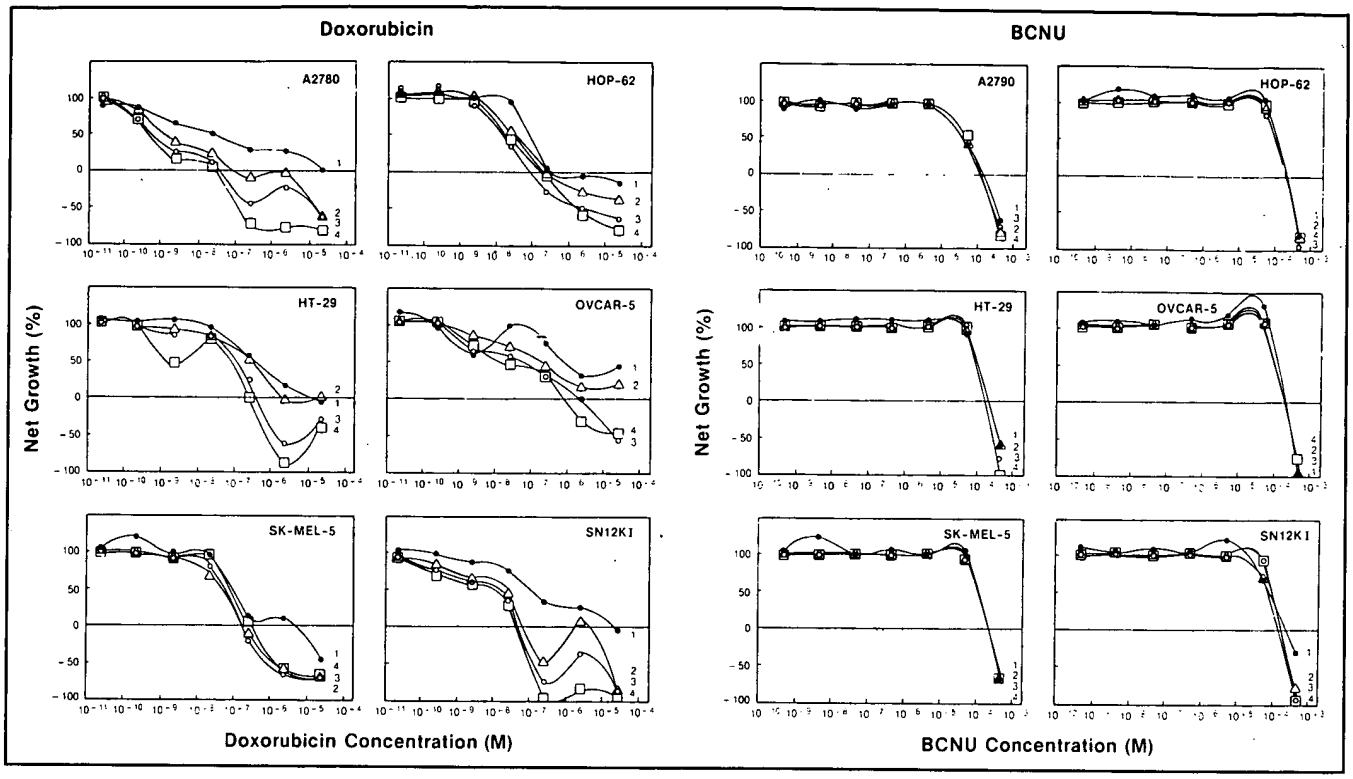

Fig 2. Dose-response curves for six cell lines incubated with doxorubicin or BCNU for 1, 2, 3, or 4 days' continuous exposure.

Table 2. Cellular response as a function of drug-incubation time as determined by SRB assay*

Incubation time (days)	% response†				Responders normalized to 4-day value‡			
	GI_{15}	GI_{50}	TGI	LC_{50}	GI_{15}	GI_{50}	TGI	LC_{50}
1	89.1	68.9	45.4	21.0	96.8	77.9	59.8	35.1
2	91.5	83.1	51.7	36.4	99.5	94.0	68.1	60.9
3	91.7	85.8	69.2	52.5	99.7	97.1	91.2	87.8
4	92.0	88.4	75.9	59.8	100.0	100.0	100.0	100.0

*GI_{15} = 15% growth inhibition; GI_{50} = 50% growth inhibition; TGI = total growth inhibition; and LC_{50} = 50% cell kill.

†% of total responses (6 cell lines × 20 drugs = 120 responses) achieving each calculated parameter.

‡Total responses achieving each calculated parameter normalized to the 4-day incubation value, which is expressed as 100%.

homoharringtonine, phyllanthoside, tamoxifen, and vinblastine. The LC_{50} was achieved only with BCNU, gossypol, homoharringtonine, and tamoxifen.

Selection of Cell Lines and Inoculation Densities

Prior to inclusion of cell lines in the screening panel, their growth and compatibility with the screening model were determined. These determinations included comparison of the linearity of the SRB-generated optical density signal with the cell number in the microculture assay. Fig 3 shows representative linear signals from 5000-50 000 cells per well in the MALME-3M melanoma line and in the U251 and XF 498 central nervous system (CNS) tumor lines, measured 1 hour after inoculation. The correlation coefficient (r) was greater than .98 for all three of these lines and greater than .95 for all panel lines. In addition, cell growth in the microculture plates was characterized at a variety of cell densities (5000, 10 000, 20 000, 40 000, and 50 000 cells per well) over a 4-day period of growth. From these data, the specific inoculation density selected for each cell line was that which produced an optical density signal above the noise level of the assay and within the linear range of the SRB signal (ie, within the range of 0.2-2.0 OD units), for both the T_0 and control optical density (C) measurements.

The T_0 and control optical densities generated from SRB-stained cells are a function of cell mass and growth rate. Thus, cells with a small mass, such as the leukemias CCRF-CEM and MOLT-4, are inoculated at the relatively high densities of 40 000 and 30 000 cells per well, respectively. Furthermore, cells that divide relatively slowly (eg, XF 498 and HOP-18) are inoculated at 20 000 cells per well, while more rapidly dividing cells (eg, NCI-H460 and HCT-116) are inoculated at 5000 cells per well.

Quality Control and Reproducibility of Cell Line Performance

Once a cell line has been included as part of the panel, all data generated from the line are stored in a database for various analyses. Fig 4 shows typical examples of the control optical densities measured for three cell lines in sequential experiments. Within each experiment, the range of control optical densities from 40 microculture plates (mean of quadruplicate wells) is also included. Cell line SK-OV-3 (ovarian cancer) showed little variation of control optical density either within or between ex-

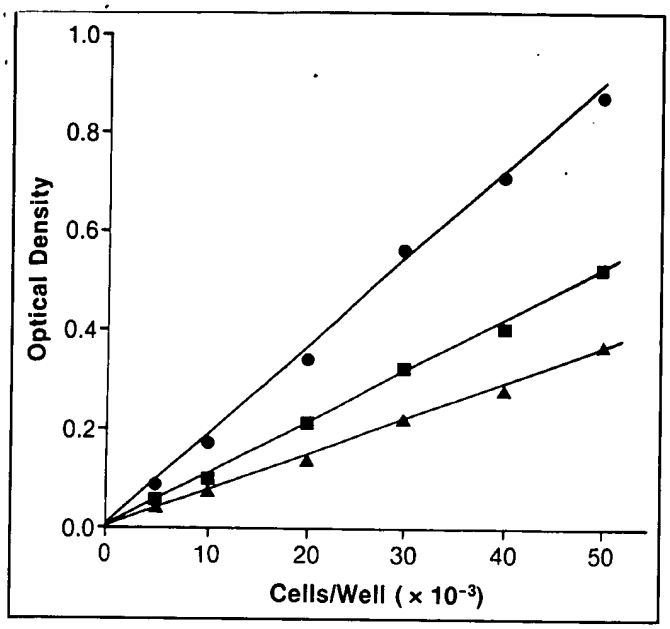

Fig 3. Three representative cell lines, showing linear relationship between increasing cell number and optical density measured by the SRB assay. ● = MALME-3M, ■ = U251, and ▲ = XF 498.

periments. The data for the SF-539 (CNS tumor) line are from two different cell inoculation densities; cultures in experiments 1, 2, 3, and 5 were inoculated at 20 000 cells per well, and the remainder were inoculated at 15 000 cells per well. The SNB-19 (CNS tumor) line initially showed more variation within and between experiments than the SK-OV-3 line, but in the latter stages, the variation appeared to decrease, perhaps due to increasing experience of the technicians handling the line. In general, the variation for a cell line, within any experiment, ranged from 0.1 to 0.4 OD units, but the interexperimental variation occasionally exceeded 1.0 OD units.

In another analysis (data not shown), the variation in the control optical density value with successive experiments was ranked from the cell line with the most reproducible values to the cell line with the least reproducible values. Examples of human tumor cell lines with the most reproducible values are SK-OV-3 (OD = 0.95 ± 0.15) and XF 498 (OD = 0.74 ± 0.13). Examples of the cell lines with the least reproducible values are SF-268 (OD = 1.3 ± 0.52); NCI-H522 (OD = 0.91 ± 0.37); HOP-18 (OD = 0.82 ± 0.36); and SK-MEL-2 (OD = 0.99 ± 0.51).

Since there was considerable variation in the resulting control optical density values from inoculation to inoculation with certain lines, it was important to determine whether the apparent sensitivity of a cell line to a drug was significantly related to the different control optical densities. Fig 5 shows the relationship between optical density and IC_{50} (n>30) measured for doxorubicin against three cell lines. The data for the KM12 (colon tumor) line showed variation of more than 1 OD unit but a consistent IC_{50}, with no relationship between optical density and IC_{50}. In our database on 64 cell lines, only five lines showed a significant relationship between optical density and IC_{50} (r>.7; P<.05); an example is the melanoma cell line UACC-62 (r = .77; P = .0001).

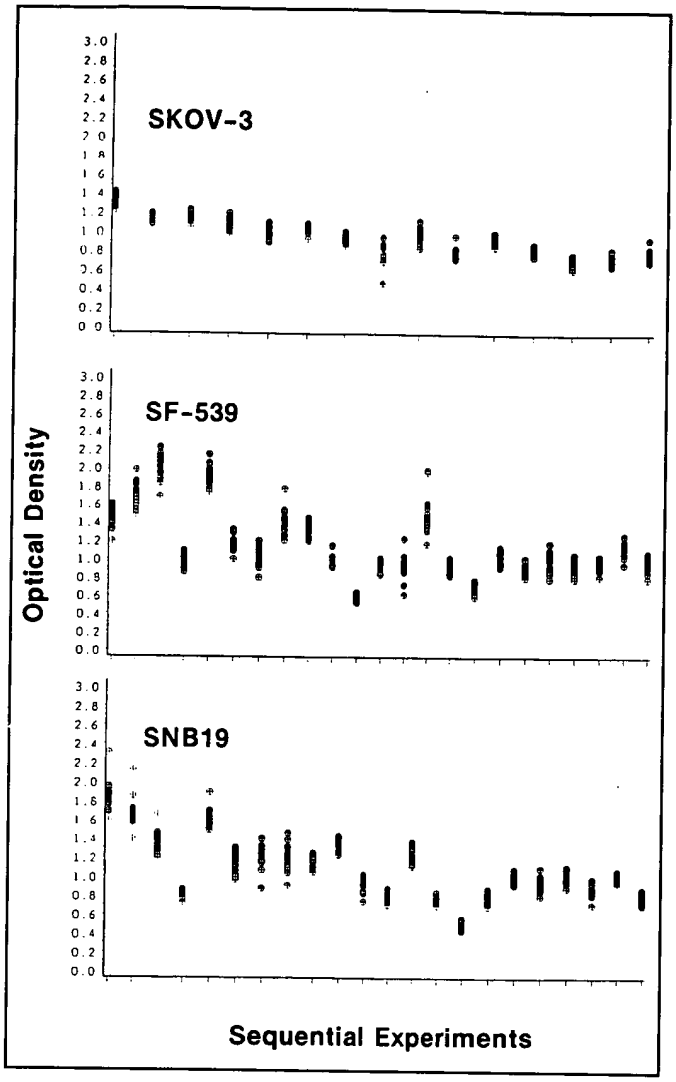

Fig 4. Range of control optical densities measured for three cell lines (SK-OV-3, SF-539, and SNB-19) in each experiment and between sequential experiments, at the rate of two experiments per week.

Reproducibility of Screening Assay

Fig 6 gives representative illustrations of the experimental variation in data obtained with the three types of assay (MTT, XTT, and SRB) using a 6-day continuous incubation of melphalan with the U251 CNS tumor line, which was independently grown, plated, and tested by 13 technicians. This experiment was repeated for three cell lines, six agents, and two incubation periods. All three assays, but particularly MTT and SRB, gave good agreement in the dose-response profiles. Generally, the greatest variations were observed at concentrations with intermediate cytostatic activity (ie, 20%-75% T/C, rather than 100% or 0%-5%).

Fig 7 shows examples of the experimental variation in doxorubicin IC_{50} values for sequential experiments with different cell lines. The IC_{50} values for most lines were highly consistent. For example, reproducibility was good, showing only occasional discrepancies in the doxorubicin IC_{50} values for the A498 renal tumor line and the COLO 205 colon tumor line.

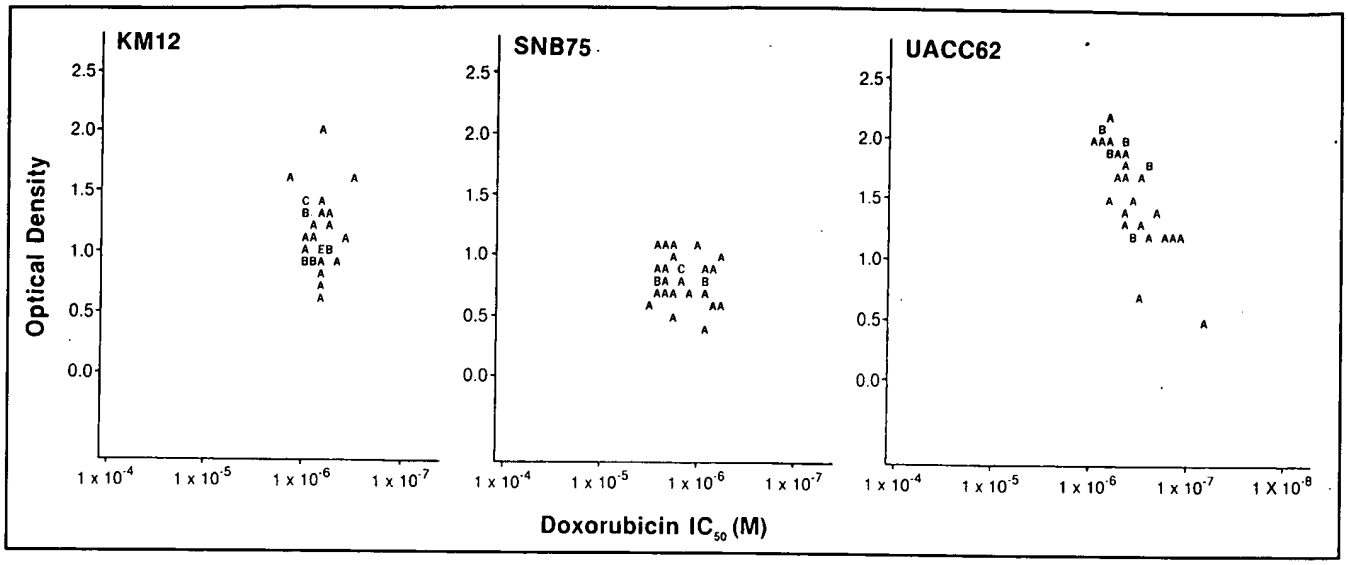

Fig 5. Correlation between control optical density and doxorubicin IC_{50} (*M*) for three cell lines (KM12, SNB-75, and UACC-62). A = one observation, B = two observations, C = three observations, E = five observations.

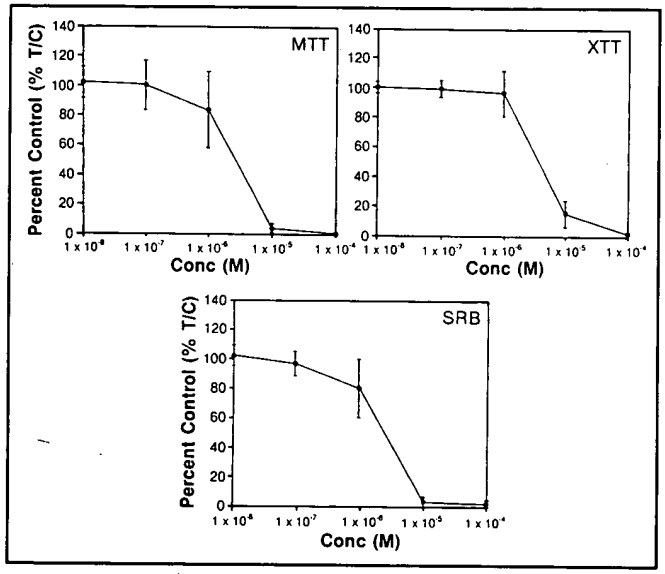

Fig 6. Percent variation (± SD) in dose-response curve for melphalan incubated with U251 cells for 6 days. Cells were grown, plated, and tested by 13 technicians using the MTT, XTT, or SRB assay. Conc = concentration.

Certain lines gave somewhat less consistent IC_{50} values. For example, the data for the K-562 leukemia line suggested initial technical handling problems resulting in poor reproducibility. As the technicians gained experience with the particular lines, however, the reproducibility of measurements of doxorubicin IC_{50} values were markedly improved. A few other lines, such as SN12KI, showed greater variation overall in the measured IC_{50} values.

The reproducibility of IC_{50} values for all cell lines in the panel was ranked. As representative examples, the cell lines showing the greatest consistency were A498 [mean $IC_{50} = 8.70 \times 10^{-7}$ *M*, 95% confidence limits (CL) = 1.33×10^{-6} *M* to 5.17×10^{-7} *M*]; COLO 205 (mean $IC_{50} = 5.1 \times 10^{-7}$ *M*, 95% CL = 8.1×10^{-7} *M* to 3.21×10^{-7} *M*); and SK-MEL-2 (mean $IC_{50} = 7.56 \times 10^{-7}$ *M*, 95% CL = 1.21×10^{-6} *M* to 4.73×10^{-7} *M*).

The cell lines showing the least consistency were NCI-H226 (mean $IC_{50} = 6.52 \times 10^{-7}$ *M*, 95% CL = 9.71×10^{-6} *M* to 4.38×10^{-8} *M*); DLD-1 (mean $IC_{50} = 7.36 \times 10^{-7}$ *M*, 95% CL = 1.17×10^{-5} *M* to 4.64×10^{-8} *M*); and CCRF-SB (mean $IC_{50} = 8.5 \times 10^{-8}$ *M*, 95% CL = 1.68×10^{-6} *M* to 4.30×10^{-9} *M*). This continuous evaluation and comparison of cell line performance over time has proved to be a useful approach to identify intractable lines for elimination from the panels or to identify technical problems leading to irreproducible results for certain lines in the screening assay. For example, erratic performance was detected with DLD-1, which was found to be due to cross-contamination of DLD-1 with NCI-H460 in the laboratory. NCI-H460 is a cell line identical to DLD-1 in appearance but more sensitive to doxorubicin (mean $IC_{50} = 5 \times 10^{-8}$ *M*). In some instances, the source of erratic behavior of particular lines could not be resolved. An example is CCRF-SB, which was dropped from the panel because of highly variable responsiveness.

Discussion

From the pilot-screen results described here, we reached a consensus that it was feasible to operate the full screen on the desired scale, using the SRB assay, the selected assay parameters, and the quality control and other operational procedures described. During November and December of 1989, after a series of reviews of the developmental status and the pilot-screening operations, a similar consensus was reached by several groups, including an Ad Hoc Expert Advisory Committee, the National Cancer Advisory Board, and the Division of Cancer Treatment's Board of Scientific Counselors (*12,13*). In addition, the specific recommendation was made that the screen had reached a sufficient level of refinement that it should be placed immediately into operational status, using the current 60-cell line panel. We have adopted that recommendation and do not anticipate further additions or substitutions to the present cell line panel in the immediate future. The screening of a large backlog of submitted compounds that have accrued at NCI

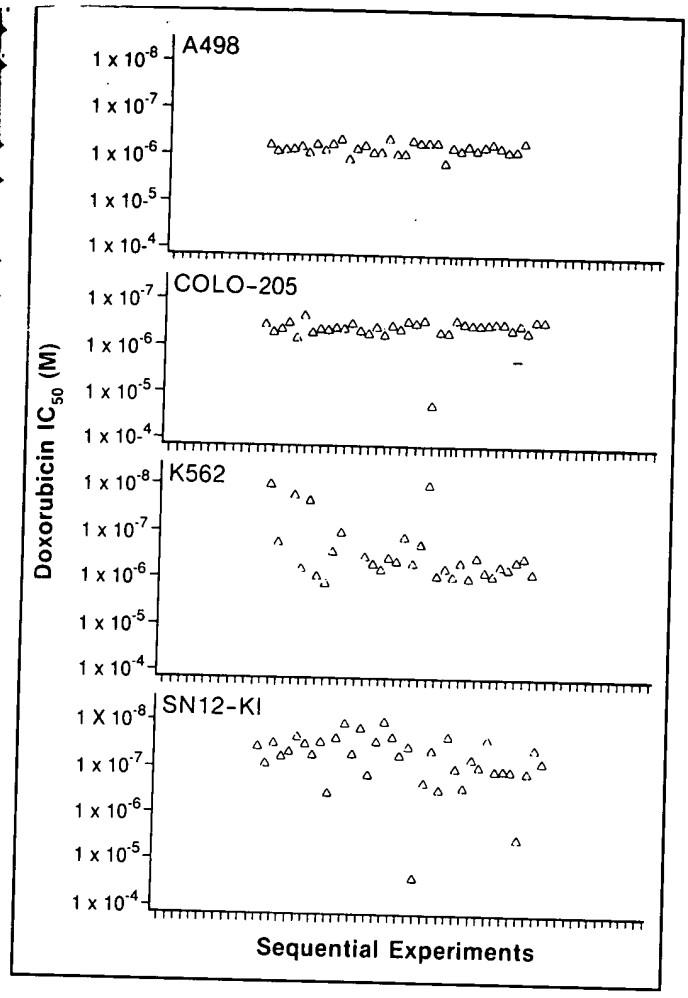

Fig 7. Representative variations in IC_{50} values for doxorubicin in four cell lines (A498, COLO 205, K-562, and SN12KI) over 4 months of sequential testing at the rate of two experiments per week, in the screening protocol.

during the developmental and pilot phases of the new screen is now under way. Selected acquisition of new compounds from suppliers worldwide for screening by the NCI program has resumed.

Currently, we are testing samples at the rate of 400 per week against the 60-line panel, using five sample dilutions, with each cell line-dilution combination being performed in duplicate wells. Appropriate measures of control (nondrug) well values and T_0 values are also obtained. The testing of each compound requires the equivalent of ten 96-well microtiter plates.

The minimum amount of compound required for screening can be readily estimated by submitters; each full test requires approximately 40 mL (0.04 L) of cell-culture medium containing two times the highest test concentration desired. For example, for a routine upper test concentration limit of 10^{-4} mol/L, the minimal amount (grams) of compound can be calculated by: molecular weight $\times 10^{-4} \times 0.04 \times 2$.

The fully operational screening laboratory and the laboratory for sample preparation together occupy 8051 sq ft of space, containing 50 laminar-flow, cell-culture hoods; 12 automated spectrophotometric plate readers; and 20 microcomputers. The screening laboratory employs 44 technical staff supervised by two PhD scientist/managers. The maximum screening rate that can currently be accomplished is approximately 400 compounds per 5-day work week, or 20 000 compounds per year. Based on current expenditures, the estimated cost to screen one compound is $200-$300.

The possibility for further increases in screening capacity, within current resources, also appears feasible as more of the routine procedures are automated. Moreover, future expansions of the cell line panel could be easily incorporated, albeit with a concomitant impact on screening capacity. For example, if the appropriate cell lines were available to expand the panel, screening operations could be performed at about half the present rate (eg, 10 000 compounds per year) on a panel about twice the present size (eg, 120 lines). Further acquisition and development of suitable lines for future addition or replacement of existing lines in the panel continues.

The question of panel and subpanel size is an important one that needs further resolution. Statistical considerations suggest that each of the disease-related subpanels should contain at least 10 representative cell lines to allow reliable determinations of subpanel-specific activity (Rubinstein L, et al: unpublished data). Nevertheless, any future decision about further expansions or other substantial modifications of the cell line panel will await sufficient experience and evaluation of the present 60-line panel.

The screening of each compound generates a voluminous amount of data, which present unique challenges for display and analysis. The further development and refinement of data management and analysis support technology for the screening program continue to be areas of intense developmental effort. Currently, suppliers of compounds will receive the screening data in a variety of investigational formats (*14*). A tabular presentation of all of the mean optical density values will be provided, in addition to the entire series of dose-response curves, plotted for each disease-related subpanel. Also included will be a series of mean graph displays of relative cellular responsiveness with respect to three different mean response parameters—GI_{50}, TGI, and LC_{50}. The derivation of the mean graph display and examples of some unique applications, such as the COMPARE pattern-recognition algorithm, have been described elsewhere (*14,15*). Finally, the data report package will also include a new data display and analysis procedure called the "dose-response matrix." In this analysis, the relative cellular responses are evaluated and compared over the entire dose-response range rather than just the midpoints. Thus, a comprehensive, detailed package of data is returned to the suppliers following testing of their compounds in this screen.

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Effects of a Low-Fat Diet on Levels of Oxidative Damage to DNA in Human Peripheral Nucleated Blood Cells

Zora Djuric, Lance K. Heilbrun, Bruce A. Reading, Allison Boomer, Frederick A. Valeriote, Silvana Martino*

Fat in the diet has been associated with increased breast cancer risk. In this study, blood samples were obtained from 21 women at high risk for breast cancer who had been randomly assigned to either a nonintervention diet or a low-fat diet. Oxidative damage was examined in the DNA from nucleated peripheral blood cells. The levels of oxidized thymine, specifically 5-hydroxymethyluracil, were threefold higher in the nonintervention diet group than in the low-fat diet group. Without regard to diet arm, there also was a significant linear relationship between daily total fat intake and 5-hydroxymethyluracil level. These results suggest that oxidative damage to DNA may be a marker of dietary fat intake. In addition, oxidative DNA damage may be a mechanistic link between fat in the diet and cancer risk, since such damage is associated with the process of tumor promotion. [J Natl Cancer Inst 83:766–769, 1991]

Epidemiological studies have suggested an association between increased fat intake and breast cancer risk (*1,2*). In an effort to modulate breast cancer risk in women at high risk for breast cancer, we initiated an intervention study that randomly assigned high-risk women to either a nonintervention diet or a low-fat diet. The diet of each woman is being closely monitored, and potential markers of fat intake and cancer risk are being established.

One intermediate marker of breast cancer risk may be oxidative damage to the DNA. Such damage may play a role in tumor promotion (*3*) and can be elicited by numerous endogenous processes, including lipid peroxidation (*4*). When DNA is exposed to oxidants, thymine residues appear to be highly susceptible to oxidation (*5,6*). Of the thymine oxidation products,

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Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth

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NEOVASCULARIZATION is critical for the growth of tumours[1–3] and is a dominant feature in a variety of angiogenic diseases[4] such as diabetic retinopathy, haemangiomas, arthritis and psoriasis. Recognition of the potential therapeutic benefit of controlling unabated capillary growth[5] has led to a search for safe and effective angiogenesis inhibitors. We report here the synthesis of a family of novel inhibitors that are analogues of fumagillin, a naturally secreted antibiotic[6] of *Aspergillus fumigatus* fresenius. We first isolated this fungus from a contaminated culture of capillary endothelial cells. Purified fumagillin inhibited endothelial cell proliferation *in vitro* and tumour-induced angiogenesis *in vivo*; it also inhibited tumour growth in mice, but prolonged administration was limited because it caused severe weight loss. Synthesis of fumagillin analogues yielded potent angiogenesis inhibitors ('angioinhibins') which suppress the growth of a wide variety of tumours with relatively few side-effects.

During routine culturing of capillary endothelial cells we found a fungal contamination that produced a local gradient of endothelial cell rounding (Fig. 1). Cells that were only a few cell diameters away were normally spread, suggesting that the fungus was specifically inducing cell rounding rather than causing toxicity. Other fungal contaminants in our experience had produced total cell detachment and cell death. Cell rounding was in itself interesting as endothelial cells must spread in order to proliferate, even when grown in the presence of saturating amounts of soluble mitogens[7,8]. Also, other angiogenesis inhibitors cause endothelial cell rounding as part of their action *in vivo*[9].

We isolated this fungus and identified it as *Aspergillus fumigatus* fresenius. Conditioned medium from fungal cultures was found to have potent endothelial cell-rounding activity and to inhibit angiogenesis in the growing chick chorioallantoic membrane. The active fraction was purified from large-scale fungal cultures and identified as fumagillin (Fig. 2), an antibiotic used to treat amoebiasis in humans[10]. Purified fumagillin completely inhibited endothelial cell proliferation in the presence of saturating levels of basic fibroblast growth factor (half-maximal inhibition of human umbilical vein endothelial cells at 0.5 ng ml^{-1}). Cell rounding, as in our original contaminated cultures, was induced at concentrations greater than 10 μg ml^{-1}. Angiogenesis was inhibited in the chorioallantoic membrane model at concentrations of fumagillin above 2 μg per chorioallantoic membrane. Fumagillin also suppressed tumour-induced neovascularization in the mouse dorsal air sac (Fig. 3), but its effectiveness as an inhibitor of tumour growth was limited because it produced severe weight loss. This side-effect probably explains the low anti-tumour effect of fumagillin in humans reported previously[11].

We therefore set out to design fumagillin analogues that would retain the potent anti-angiogenic activity of fumagillin without its side-effects. Alkaline hydrolysis of fumagillin yields fumagillol, from which over 100 derivatives were synthesized and tested. Among these analogues we identified a subset of compounds that represent a new class of angiostatic antibiotics which we term angioinhibins, one of the most potent of which is *O*-(chloroacetylcarbamoyl)fumagillol or AGM-1470 (Fig. 2). This angioinhibin produced half-maximal cytostatic inhibition of endothelial cell proliferation at ~10 pg ml^{-1}, which is 50 times more active than the fumagillin parent. Cytotoxicity (reduction of cell number below the initial plating density) was only observed at much higher concentrations (>1 μg ml^{-1}). Although the sensitivity of a normal non-endothelial cell line (human embryonic lung fibroblasts) was not significantly different from human umbilical vein endothelial cells, doses of AGM-1470 had to be at least ten times higher for comparable inhibition of the

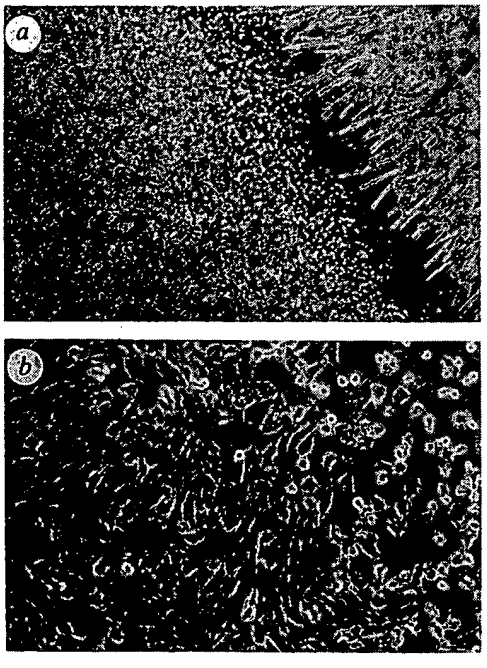

FIG. 1 *a*, A contaminated culture of bovine capillary endothelial cells showing fungal hyphae at the right. *b*, Higher magnification of *a* showing an apparent diffusion gradient resulting in cell detachment and rounding nearest the edge of the fungal colony; cells only a few cell diameters away have normal morphology.

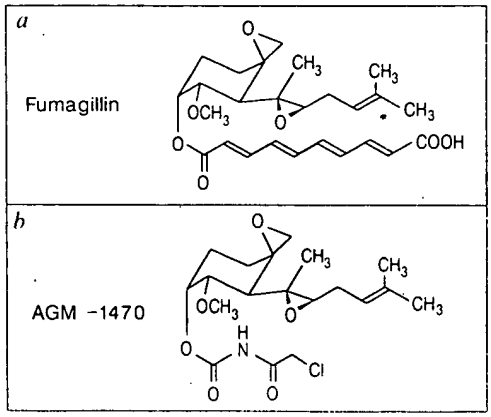

FIG. 2 Structure of *a*, fumagillin, the parent compound isolated from the culture contaminant described in Fig. 1, and *b*, the synthetic analogue AGM-1470, a related angioinhibin used in these antitumour studies.

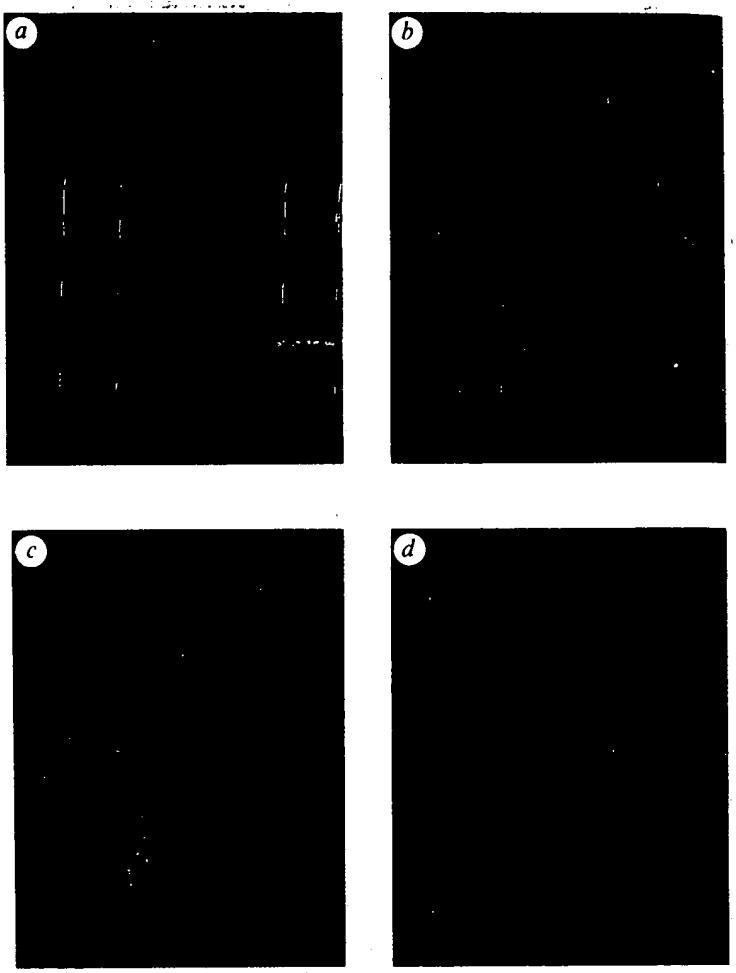

FIG. 3 Inhibition of tumour-induced angiogenesis in the subcutaneous dorsal air sac of the mouse by fumagillin. Chambers containing Millipore filters (0.45 μm) were filled either with saline (*a*, *c*) or 5×10^6 ascites sarcoma-180 cells in saline (*b*, *d*) and implanted in dorsal air sacs created surgically in mice. Animals containing the implanted chambers were treated systemically with fumagillin (100 mg kg^{-1}, subcutaneously) for 3 days (*c*, *d*). On day 4, angiogenesis within the subcutaneous fascia of fumagillin-treated animals (*c*, *d*) was compared with that of controls (*a*, *b*).

human tumour cell lines (squamous cell HSC-1, colon COLO-205, leukaemia HL-60 and squamous cell LS-180).

AGM-1470 also inhibited growth of solid tumours when it was delivered systemically in mice. These tumours included Lewis lung carcinoma (Fig. 4*a*, *b*), B16 melanoma (Fig. 4*c*, *d*), M5076 reticulum cell sarcoma, Meth A sarcoma, colon 26 carcinoma, and Engelbreth-Holm Swarm sarcoma (and others not shown). There was a sustained inhibition of tumour growth

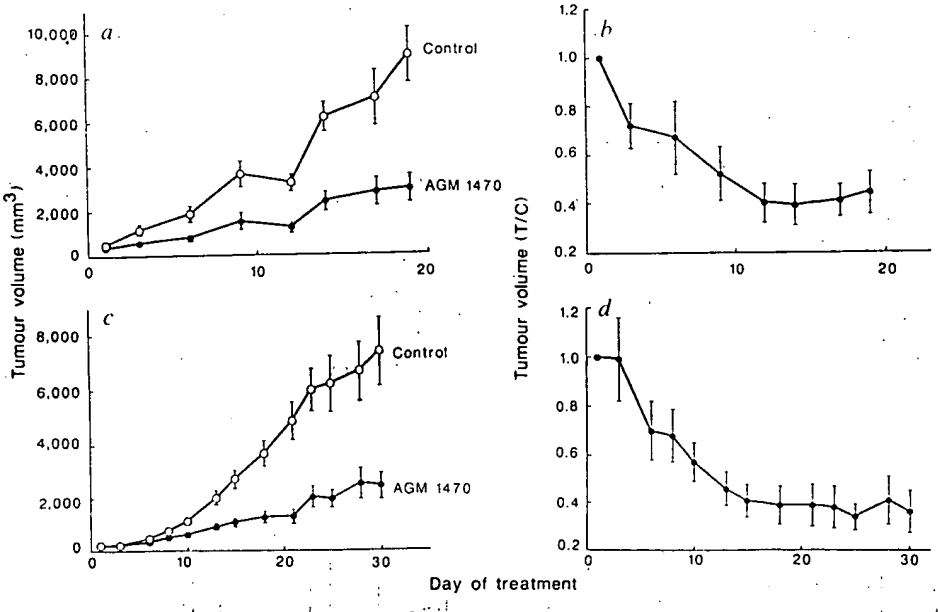

FIG. 4 Inhibition of growth of Lewis lung carcinoma (*a*, *b*) and B16 melanoma (*c*, *d*) by systemic administration of AGM-1470. Tumour cells (1×10^6) were inoculated subcutaneously in 0.1 ml saline in the same position along the dorsal midline in C57B1/6 mice. Treatment was not initiated until tumours were at least 100 to 250 mm^3 in volume. Tumours of similar size were matched for use in control and experimental groups. AGM-1470 was administered subcutaneously at a remote site at 30 mg per kg in body weight in saline once every other day. Tumours were measured in two dimensions and volume was calculated using a standard formula: width^2 *x* length ×0.52. *T*/*C* = tumour volume of treated/mean tumour volume of control (untreated) for each day. Lewis lung carcinoma, *N* = 29 animals per group; B16 melanoma, *N* = 16 animals per group. Error bars indicate standard error of the mean.

regardless of whether administration of AGM-1470 was started one day after tumour inoculation (data not shown) or once the tumour had become fully established (when tumour volume exceeded 100 mm^3) (Fig. 4). Whereas the application of fumagillin in animals was restricted by their unacceptable weight loss (>15% of starting weight), animals treated with effective doses of AGM-1470 gained weight slowly but consistently. Daily therapy against the tumour was unnecessary in any animal, with less frequent regimens of once every three days, for example, being highly successful (in the case of M5076-bearing animals, survival time increased by up to 260% over untreated controls). Yet cells from the M5076 tumour cell line, which were very sensitive to AGM-1470 *in vivo*, were found to be refractory to AGM 1470 *in vitro*. These tumour cells *in vitro* required 100 times more AGM-1470 than vascular endothelial cells for comparable inhibition of growth. Taken together, these results strongly suggest that AGM-1470 exerts its anti-tumour effects primarily by acting on the tumour vasculature. This hypothesis is further supported by the finding that AGM-1470 did not prolong the survival time of mice injected intraperitoneally with P388 leukaemia. This tumour grows in an ascites (non-solid) form and should therefore be less dependent on neovascularization for its development.

Angioinhibins, such as the one reported here, represent a unique class of angiostatic antibiotics. Unlike other angiogenesis inhibitors, angioinhibins are not steroid[12,13], polysaccharide[13,14], retinoid[15] or peptide[15–17] structures. There are two other microbial products with angiostatic activity: one is a sulphated polysaccharide peptidoglycan complex[18] whose complete structure is still unknown; the structure of the other has been determined[19] but it is cytotoxic and its anti-tumour effects have not been reported.

Our new class of angiogenesis inhibitors, then, can suppress tumour growth without the usual toxic side-effects associated with conventional chemotherapy drugs. Specifically, we never observed hair loss, intestinal disturbance or infection although we administered AGM-1470 subcutaneously at 30 mg kg^{-1} every other day for >100 days (which is about one sixth of the life of a mouse). This absence of side-effects could be critical in treatment of tumours and other angiogenic diseases which may require long-term therapy in much the same way as diabetes and malaria do now. An example of prolonged anti-angiogenic therapy for a non-neoplastic disease has been reported in the treatment of haemangioma with alpha-interferon[20]. These findings in conjunction with ours provide a glimpse of what a cancer therapy based on anti-angiogenesis might be in the future. □

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No T-cell tyrosine protein kinase signalling or calcium mobilization after CD4 association with HIV-1 or HIV-1 gp120

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THE T lymphocyte surface protein CD4 is an integral membrane glycoprotein noncovalently associated with the tyrosine protein kinase $p56^{lck}$ (reviewed in ref. 1). In normal T cells, surface association of CD4 molecules with other CD4 molecules or other T-cell surface proteins, such as the T-cell antigen receptor, stimulates the activity of the $p56^{lck}$ tyrosine kinase, resulting in the phosphorylation of various cellular proteins at tyrosine residues[2–4]. Thus, the signal transduction in T cells generated through the surface engagement of CD4 is similar to that observed for the class of growth factor receptors possessing endogenous tyrosine kinase activity[5]. As CD4 is also the cellular receptor for the human immunodeficiency virus (HIV)[6,7], binding of the virus or gp120 (the virus surface protein responsible for specific $CD4^+$ T-cell association) could mimic the types of immunological interactions that have previously been found to stimulate $p56^{lck}$ and trigger T-cell activation pathways. We have evaluated this possibility and report here that binding of HIV-1 or the virus glycoprotein gp120 to $CD4^+$ human T cells fails to elicit detectable $p56^{lck}$-dependent tyrosine kinase activation and signalling, alterations in the composition of cellular phosphotyrosine-containing proteins, or changes in intracellular Ca^{2+} concentration.

The results presented in Fig. 1 demonstrate that crosslinking surface CD4 molecules with anti-CD4 monoclonal antibodies results in the stimulation of CD4-associated $p56^{lck}$ tyrosine protein kinase activity leading to the phosphorylation of T-cell proteins. Parallel immunoblots detecting $p56^{lck}$ or CD4 show that during the course of these experiments the abundance of $p56^{lck}$–CD4 complexes was not altered. These results imply that the specific activity of the CD4-associated $p56^{lck}$ was transiently increased around four- to fivefold as a consequence of antibody-mediated CD4 crosslinking. The results of anti-phosphotyrosine immunoblots indicate that cell proteins can be phosphorylated on tyrosine residues in response to the same CD4 crosslinking treatment. In addition, crosslinking of CD4 stimulated Ca^{2+} mobilization after the activation of $p56^{lck}$, and also led to the rapid appearance of interleukin-2 receptor α subunit (IL-2Rα) and transferrin receptor on the surface of the T cells.

To evaluate HIV interactions, purified HIV-1 (strain III-b) was added to the quiescent T cells on ice and binding was monitored by flow cytometry measuring the capacity of the virus to block subsequent binding of fluorescein-conjugated monoclonal antibody Leu3A. HIV-1 could saturate the available surface Leu3A sites within 30 min (Fig. 2*a*), but these conditions did not result in detectable HIV endocytosis and did not affect the subsequent binding of monoclonal antibody OKT4, which recognizes another CD4 surface epitope distinct from the HIV-binding domain. We found that HIV binding and prolonged

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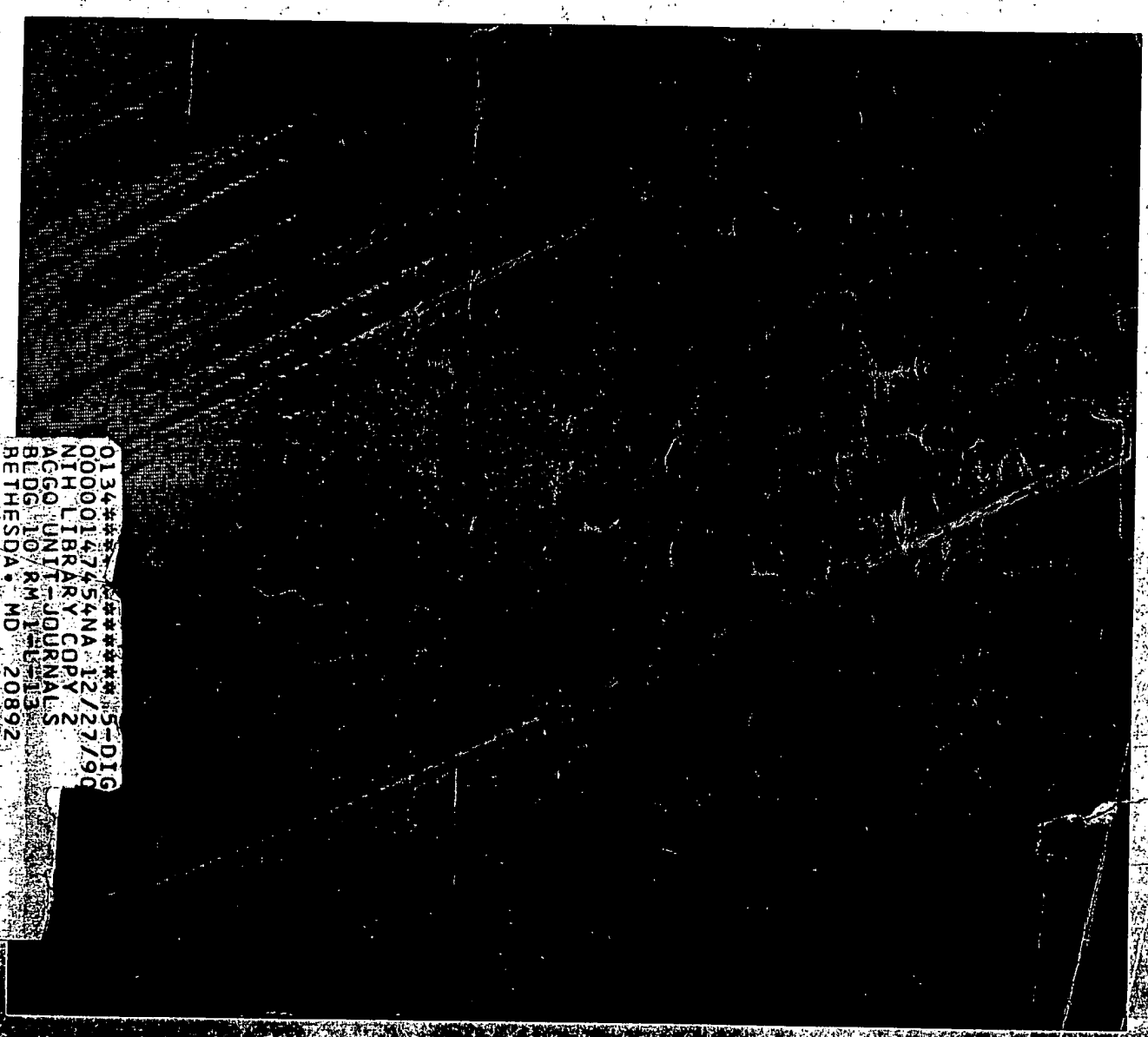

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Brightness changes in Sun-like stars

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